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Principal Investigator:
George A. Feigen,
Department of Physiology,
Stanford University,
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PREFACE

The bacterial toxin, streptolysin O, historically known as a potent hemolytic agent, has been shown more recently to be toxic for the mammalian heart *in vitro* as well as *in vivo*. There is mounting evidence that the toxin is involved in the pathogenesis of the focal cardiac lesions of rheumatic fever. During the course of this disease, the anti-streptolysin O antibody content of the patient's serum is characteristically high, and the titer of this antibody in the serum has long been used as an indication of the severity and course of the disease. This fact is of particular interest in light of recent indications that rheumatic fever may be a hyperimmune or autoimmune disorder.

These considerations have led to the present study of the relationship between the cardiotoxic and immunologic properties of streptolysin O. The primary thesis underlying this work is that the direct action of the toxin on the heart may be exaggerated by an additional reaction between the toxin and the specific anaphylactic, anti-toxin antibody bound to the cardiac tissues.

The present work is logically divided into three sections. In Part I a detailed analysis of the cardiotoxic properties of streptolysin O is presented, and an hypothesis concerning the mode of action of the toxin on the normal heart is developed. Before

the anaphylactic properties of streptolysin O could be investigated, it was necessary to study the phenomenon of cardiac anaphylaxis in the guinea pig so as to establish a set of experimental conditions under which a quantitative analysis of the process could be carried out with the toxin. Part II, then, provides the ground rules for the studies of Part III, which establish the antigenic nature of streptolysin O, and demonstrate that the direct reaction between the toxin and the heart tissue can occur simultaneously with the reaction between the toxin and the specific tissue-bound antibody.

PART I. Cardiotoxicity of Streptolysin O

INTRODUCTION

General Considerations

It has been known since the time of Roux and Yersin (1) and Kitasato (2) that certain bacterial species are sources of substances that are directly toxic to host organisms. These substances, the bacterial toxins, are antigenic, poisonous materials of high molecular weight that give rise to characteristic symptoms, including death, when injected into susceptible animals (3).

The bacterial toxins are conventionally classified as endotoxins or exotoxins according to whether they are found inside or outside the parent organism. However, van Heyningen (3) prefers what he considers to be a more significant and unequivocal system which classifies the toxins according to whether they are derived from Gram-positive or Gram-negative bacteria. Such a system, which still roughly divides the toxins into endotoxins and exotoxins, is based on the following points:

(a) The Gram-positive toxins are found outside the parent cells. A typical Gram-positive toxin is excreted during the phase of active growth of the organism and reaches its highest concentration at the time growth reaches its maximum.

On the other hand, the toxins from Gram-negative organisms appear to be structural components of the bacterial cells, and some investigators (4, 5) have shown that washed dead

bodies of a number of Gram-negative organisms are toxic, whereas those of a Gram-positive bacteria are not.

(b) Antibodies to Gram-positive toxins are capable of completely neutralizing their activity; antibodies to Gram-negative toxins can only neutralize a fraction of their activities.

(c) The Gram-positive toxins are generally more toxic than the Gram-negative toxins.

(d) The Gram-positive toxins are generally less heat-stable than the Gram-negative toxins.

(e) The Gram-positive toxins can be toxoided, that is, made non-toxic but still capable of stimulating the production of antibodies that neutralize the toxins. Most Gram-negative toxins cannot be toxoided in the ordinary way.

(f) The Gram-positive toxins that have been purified are simple proteins, whereas most Gram-negative toxins appear to be polymolecular phospholipid-polysaccharide-protein complexes.

(g) The pharmacology of the Gram-positive toxins is generally quite specific, i.e., the symptoms produced by each toxin are characteristic of that material; the symptoms produced

by Gram-negative toxins are more nearly the same.

A group of toxins, which, according to these criteria, must be classified as exotoxins, are the cytolytic toxins of bacterial origin (6). This group is composed of a number of toxins from a variety of organisms, and includes the two hemolytic toxins of *Streptococcus pyogenes:* streptolysin O and streptolysin S. The substances belonging to this group have certain features in common, some which classify them under the general heading of exotoxins, and others which place them in the specific category of cytolytic toxins: (i) all of them are found extracellularly; (ii) most of them are proteins [streptolysin S, or its active moiety, appears to be a relatively small polypeptide (7, 8, 9)]; (iii) all of them are lytic for erythrocytes when tested *in vitro,* and some of them are the most potent hemolytic agents known; (iv) they are lytic for a variety of other cell types; and, finally, (v) most of them are lethal for laboratory, and presumably for other, animals, the lethal dose being considerably larger than that of the classical exotoxins (6).

Several of these cytolytic toxins are further categorized into a group referred to as the oxygen-labile hemolysins (3). Streptolysin O is the prototype of this group of closely similar toxins, which are produced by Gram-positive bacteria and include pneumolysin, tetanolysin, cereolysin, and the θ-toxin of *C. welchii.* The oxygen-labile hemolysins have the following properties in common: (i) they are active only in

the reduced state and are reversibly inactivated by mild oxidizing agents; (ii) they are inhibited by cholesterol; (iii) any one of the hemolysins is inhibited by the specific antitoxin to any of the others.

Early Observations

In 1897, Bordet (10) drew attention to the fact that there generally was hemolysis in the blood of rabbits which had died of streptococcal infection (11). This observation led to attempts to isolate the responsible hemolytic substance by studying streptococcal-induced hemolysis *in vitro*.

By growing a virulent streptococcus in heated rabbit serum and filtering the resulting cultures, Besredka (12) obtained filtrates that hemolysed rabbit red cells as well as those of several other species including guinea pig and man.

Ruediger (11) prepared his material, which he called "streptocolysin", in much the same way as did Besredka, but with some refinements. His filtrates, which hemolysed both washed and unwashed cells of a variety of animals, were non-dialysable, heat-labile (70°C for 2 hours), and different from the immune hemolysins in that they did not require the combined action of an intermediary body and complement.

Ruediger noted that washed cells were considerably less resistant to hemolysis than were unwashed cells, and conjectured that there was

something in the sera of these animals -- an anti-hemolysin -- which protected the red blood cells against streptocolysin. He supported this idea by showing that both normal and heat-treated chick serum inhibited hemolysis of chick and rabbit red cells by the streptococcal filtrates. In further studies (11, 13), Ruediger found that his hemolysin preparation behaved as though it were composed of two active groups tightly bound together: a haptophore group and a toxophore group. By means of the haptophore group, which was neutralized by chick serum, the toxin attached to the cell in such a way that the toxophore group, which was inhibited by $ZnCl_2$ could exert its destructive effect on the cell. Although the hemolysin could be bound to the red cells at 6°C so firmly that it could not be removed even by repeated washings, hemolysis took place only after the temperature was raised to near 37°C. In his second paper (13) Ruediger called his hemolytic filtrate "streptolysin"; the fact that this hemolysin was heat-stable up to 65°C suggests that the active principle must have been what is now termed streptolysin S.

The term "hemotoxin" was proposed by Pribram (14) to distinguish the bacterial lysins which exhibited antigenic properties from other substances of bacterial origin which were hemolytic but not antigenic, and from the immune hemolysins. The hemotoxins were thus defined as being (i) directly toxic for red cells and (ii) antigenic, in that, when injected into animals, they induced the formation of a neutralizing hemotoxin. The hemolysins, on the other hand, were characterized as sensitizing antibodies induced by the injection of a primarily non-toxic

antigen (red blood cells), and, unlike the hemotoxins, their species-specific hemolytic action was dependent upon the cooperative mechanism of the sensitizing hemolysin and complement.

In 1920, Meader and Robinson (15) reported the results of their studies on the streptococcal hemotoxin in which they found that the hemolytic activity was neither in nor on the bacterial cell, but was free in the culture medium. They made a very important observation to the effect that the hemolytic potency of their preparations was decreased following centrifugation or shaking. It is likely that this lability was due to oxidation of the hemotoxin, which was aggravated by these treatments.

Neill, who had previously studied the oxidation-reduction reactions of hemoglobin, applied the principles derived from these studies to the oxidation and reduction of bacterial hemotoxins (16, 17, 18, 19). Based on his experience with the pneumococcus hemotoxin, the hemotoxin of the Welch bacillus, tetanolysin, and streptolysin, Neill established a number of properties common to the so-called oxygen-labile hemotoxins:

(a) the active hemotoxin is converted by oxidation to a product devoid of hemolytic activity.

(b) the oxidation product of the hemotoxin can be converted back to the original hemolytic substance by reduction with chemical

or biological reducing agents.

(c) the active hemotoxin produced by the reduction of the inactive oxidation product is identical to the original, active, reduced hemotoxin. It exhibits the same activity, possesses the same degree of thermolability, and, most importantly, is neutralized by the same specific antibody.

(d) the hemotoxin, both in its reduced and oxidized forms, is irreversibly inactivated by the same heat treatment; the heat-inactivated toxin cannot be reactivated by reduction.

In their study of streptolysin, Neill and Mallory (19) found that this hemotoxin was extremely oxygen-labile, losing its activity rapidly in the presence of free air, and deteriorating slowly even when stored in sealed vials.

Neill and Mallory (19) also noted that there was no convincing evidence in the literature that streptolysin was an antigen, and it was generally accepted that the antistreptolysin titer of the sera of normal animals could not be increased by immunization with the toxin. This view was still current in 1932 when Todd (20) demonstrated that the lack of antigenicity was caused by the serum used in the usual method of preparation of streptolysin. Hemolytic streptococcal filtrates, prepared in serum-free media (19) behaved like any ordinary antigen.

Todd showed that the presence of serum in the culture medium affected the hemolysin in unexpected ways, causing delayed hemolysis, resistance to oxidation or reduction, and inability to combine with the specific antihemolysin which readily neutralized serum-free hemolysin. In trying to explain the differences between the streptolysin produced by the two methods, Todd argued that the serum streptolysin owed its characteristics to adsorption by serum, and that streptolysin formed in serum-free cultures was in a free or unbound form and therefore exhibited different properties.

In 1934, however, Todd disproved his own theory (21). He reported that hemolytic streptococci from human infections produced two hemolysins: an antigenic streptolysin which was subject to reversible oxidation and reduction; and a streptolysin which was oxygen-stable and appeared to have no antigenic activity. He later showed (22) that these streptolysins were distinct, serologically, and named one streptolysin O to indicate its sensitivity to reversible oxidation, and the other, streptolysin S, to indicate its solubility in serum. Todd showed that the two streptolysins were neutralized by separate, unrelated antibodies. Streptolysin O was, in itself, antigenic, but streptolysin S was not antigenic when separated from the parent streptococci. When animals were inoculated with living cultures of group A hemolytic streptococci, antibodies to both toxins were formed (23).

Lancefield's division of hemolytic streptococci into groups greatly

facilitated the serological examination of streptolysins (24, 25, 26). Todd (21, 23) found that Lancefield's groups of hemolytic streptococci (group A from human infection, group C from animal infection, etc.) each produced different varieties of streptolysin which he characterized by their reactions to oxygen, heat, and pH.

In 1940, Smythe and Harris (27) published an account of their studies in which they attempted to purify their streptolysin O. Since they cultured their bacteria in serum-free medium, they found no trace of streptolysin S. They began with the broth from this culture, and, by repeated precipitation with ammonium sulfate followed by extractions with ethyl alcohol, acetic acid, and sodium bicarbonate, respectively, were able to produce a relatively pure preparation. These authors undertook an analysis of this streptolysin O, and concluded from these studies that streptolysin O was a protein, that it contained rather large amounts of sulfur, and that a part of the sulfur existed as an oxidation-reduction system of the following form:

$$-SH \rightleftarrows -S-S-$$

It appeared that, in the active form, this oxidation-reduction system was in the -SH form, since removal of -SH groups by any means resulted in inactive hemolysin.

Smythe and Harris, in characterizing their streptolysin O, noted

that cholesterol completely inhibited its hemolytic activity and this inhibition could not be reversed by the usual reactivating agents. The inhibition of the oxygen-labile hemolysins by cholesterol is an important phenomenon. It has been known for a long time that cholesterol inhibits a number of hemolytic substances (28), and, in fact, it has been suggested that one of the biological functions of cholesterol is its antihemolytic activity. All of the oxygen-labile hemolysins are inhibited by minute amounts of cholesterol in the form of an artificially prepared suspension (29), but such cholesterol suspensions do not inhibit the oxygen-stable hemolysins (3). Furthermore, cholesterol combines with, and inhibits, the oxygen-labile hemolysins only when they are in the reduced (active) state.

The inhibitory actions of cholesterol on the oxygen-labile hemolysins have been studied in relation to structure and configuration by Berliner and Schoenheimer (30). This sterol, and others that are structurally closely related (31), apparently inhibits the lytic activity of the oxygen-labile hemolysins by adsorbing them (3). Howard *et al.* (31) suggested that this inhibition was due to complex formation between the lysin and the sterol molecule, and further suggested that the lysis of red cells by streptolysin O was dependent on complex formation between the lysin and the cholesterol of the red cell membrane, presumably resulting in penetration of the membrane. Evidence for this theory is not lacking. Smythe and Harris (27) and Cohen, *et al.* (32) found that hemolysed red cells inhibited the action of the oxygen-labile

hemolysins. Smythe and Harris further observed that streptolysin O was rapidly inactivated as it acted on red cells; and Cohen, *et al.* noted that washed red cell stromata were also inhibitory, but not after their cholesterol had been extracted. These facts suggested that the inhibitory substance of hemolysed red cells was cholesterol which was known to be a normal constituent of the red cell surface. Furthermore, all cells that are sensitive to streptolysin O contain cholesterol as a constituent of their cell membranes, and all cells so far examined that lack membrane cholesterol, are insensitive to its lytic action (33, 34). The combined evidence is therefore very strong that membrane cholesterol is involved in the lytic action of streptolysin O (6).

Since Smythe and Harris (27), procedures for concentration and purification of streptolysin O have been described by a number of investigators (for example, 35, 36). Although these methods yielded products much purer than the starting materials, none of them was really satisfactory because large losses were incurred during the course of purification (37). Recently however, Halbert and his co-workers have developed methods for the relatively efficient preparation of highly purified streptolysin O (38 - 42). These methods involve precipitation of the extracellular medium with an ammonium sulfate solution, and continuous-flow electrophoresis of a buffered solution of the precipitated material followed by chromatography on a column of calcium phosphate. The lyophilized product is 100 % protein in the case of material from group A cultures, and 60% protein in the case of group C toxin.

The group A toxin has 160,000 hemolytic units per milligram protein; the group C toxin, 60,000 (43). Todd (23) reported the streptolysins from group A and group C streptococci to be identical, and Halbert (40, 41) has supported this claim with immunodiffusion studies.

Lethality of Streptolysin O

Although it was known that fatal streptococcal infection in rabbits frequently was accompanied by hemolysis, that streptolysin was fatal, was not demonstrated until sometime later. Hewitt and Todd (44) first demonstrated the lethal action on mice, and Todd and his co-workers further characterized this property in subsequent publications (35, 45). Halbert, Bircher, and Dahle (46) confirmed these results in the mouse and conducted a thorough study on the lethality of streptolysin O in the rabbit (46, 47).

In all, mice, rats, guinea pigs, rabbits, and cats, have been shown to be susceptible to the lethal effects of intravenous streptolysin O. However, these animals differ appreciably in their susceptibilities to the toxin, the unit lethal dose being approximately forty times as great for the mouse as for the rabbit (37). The lethal effect of streptolysin O is prevented (i) by allowing it to react with antistreptolysin O or cholesterol prior to injection, or (ii) by injecting the toxin in the oxidized state (37). Bernheimer observed some time ago that repeated injections of sublethal amounts of streptolysin O into mice at short intervals were followed by the development of refractoriness to a

subsequently injected lethal dose of toxin (48).

Cardiotoxicity of Streptolysin O

Bernheimer and Cantoni showed that the cellular action of streptolysin O was not limited to the lysis of blood cells. They induced systolic contracture of the isolated frog heart with solutions containing the toxin (36, 49, 50). Kellner, Bernheimer, *et al.* (51) later demonstrated that the mammalian heart was also very sensitive to this toxin by perfusing very small quantities of their material through the coronary arteries of isolated hearts of guinea pigs, rabbits, and rats. Under these conditions, permanent and irreversible cardiac standstill occurred, accompanied by a marked but transitory decrease in the flow of perfusion fluid. Although these investigators used relatively crude streptococcal extracellular preparations containing streptolysin O, they identified the role of this molecule in the observed reactions by taking advantage of the characteristic properties of the oxygen-labile hemolytic toxins (3): (i) heat lability, (ii) inactivation by cholesterol, (iii) reversible oxidation, (iv) inhibition by specific antibody.

As mentioned earlier, Halbert (38 - 42) succeeded in obtaining moderate quantities of highly purified streptolysin O from both group A and group C streptococci. In assessing the pathological significance of this pure extracellular component, Halbert, Bircher, and Dahle (52, 46) studied its cardiotoxicity for rabbits *in vivo*. Electrocardiograms,

obtained from rabbits given lethal and sublethal doses of highly purified streptolysin O, were characterized by conduction defects and ventricular automatism. Intravenous injection of several multiples of the LD_{50} was followed within 3 to 4 seconds by a rapid transition from normal sinus rhythm to ventricular arrhythmia, ventricular fibrillation, and standstill. Frequently, smaller lethal doses and sublethal doses immediately produced a pronounced but transient sinus bradycardia, followed by temporary or permanent recovery. The transient nature of this bradycardia, along with the extremely rapid effects of the toxin *in vivo*, suggested that its toxicity might result from the release of vasoactive substances rather than from the direct action of the protein itself. Accordingly, Halbert, *et al.* (47) studied the effectiveness of various pharmacological blocking agents in protecting against the physiologically active agent(s) responsible for the intravenous effects of streptolysin O. Although the several agents which exhibited protection were serotonin antagonists, there was little correlation between this property and protection. In addition, serotonin was not very toxic to rabbits and mice, so it appeared unlikely that the acute toxicity of streptolysin O could be accounted for entirely by the release of this amine.

In subsequent studies, Halbert, *et al.* (53) considered the likelihood that the *in vivo* cardiovascular changes observed were consequent to an elevated plasma potassium concentration resulting from intravascular hemolysis. They found sufficient discrepancies, however, in the relationships among hemolysis, potassium levels, and electrocardiographic

changes to render inadequate this mechanism for the rapid lethality of the toxin. Furthermore, the pharmacologic agent most protective against this lethality had no effect against hemolysis or toxic levels of KCl.

Streptolysin O and Rheumatic Fever

The complex properties of streptolysin O, as manifested in its cytotoxic, lethal, and cardiotoxic actions, make it an extremely interesting substance to study. Moreover, because of the relationship between streptococcal infections and rheumatic fever, studies of streptolysin O are of great practical importance.

It is well known that rheumatic fever is preceded by streptococcal infections, and strong evidence indicates that this microorganism causes the disease (54). Although the mechanism has not been established, it has been suggested that rheumatic fever may be the result of an unusual intoxication by some product(s) of the streptococcus (55), or that hypersensitive (56), or autoimmune mechanisms (57), are responsible for the morbidity. Whatever the mechanism, the specific cause and effect relationship between streptococcal infection and rheumatic fever indicates that a substance associated with this microorganism is etiologically involved (46).

There is considerable circumstantial evidence that streptolysin O may be the streptococcal product responsible for the pathogenesis of the cardiac lesions of rheumatic fever (46). The work of Bernheimer and

Cantoni (36), and Kellner, Bernheimer, *et al.* (51) demonstrated that streptolysin O has a direct toxic effect on the amphibian and mammalian heart *in vitro,* and Halbert, *et al.* (46) observed severe electrocardiographic disturbances in rabbits injected with the toxin. In these studies, Halbert found small focal inflammatory lesions in the ventricles of rabbits dying from the toxin injection. Earlier, Schwab, *et al.* (58) treated rabbits with certain streptococcal factors and noted a high incidence of focal cardiac lesions when the animals were injected later with culture filtrates. They presented evidence that streptolysin O was responsible for the cardiac lesions. Murphy and Swift (59) and others (60) have shown that repeated streptococcal infections cause focal cardiac lesions in rabbits. These results were confirmed by Kirschner and Howie (61), who also correlated the incidence and severity of such lesions with the antistreptolysin O titer of the serum.

The very frequent rise in antistreptolysin O titer following streptococcal infections in humans is evidence that streptolysin O is produced *in vivo* during the course of such diseases (54). Therefore, it is of extreme significance that patients with rheumatic fever tend to have higher antistreptolysin O titers than patients with uncomplicated streptococcal disease (54, 62, 63), along with higher titers of some other antistreptococcal antibodies (64, 65). This is not a non-specific increase in antibody production (66, 67).

There is some evidence (66, 67) that rheumatic patients exhibiting

hypercholesterolemia due to other diseases, seem refractory to rheumatic fever recurrences. Conversely, rheumatic patients suffering from hypocholesterolemia show increased susceptibility to rheumatic fever.

Aim of Present Work

Considering the compelling nature of these lines of evidence implicating streptolysin O in rheumatic fever and suggesting that the toxin has a direct effect on the heart, there is relatively little knowledge concerning the action of streptolysin O on the heart, *in vitro*, and none at all pertaining to its possible mode of action.

For these reasons, the present work has been undertaken in an attempt to extend and to relate the *in vitro* studies (36, 51) and the provocative *in vivo* electrocardiographic findings (46, 47, 53) by an analysis of the electrophysiological, pharmacological, and mechanical changes occurring in the isolated whole heart and separate cardiac tissues of the guinea pig pursuant to their challenge with streptolysin O.

MATERIALS AND METHODS

Reagents.

Toxin. Several preparations of streptolysin O, obtained from group A and group C streptococcal extracellular concentrates, were supplied as lyophilized powders in the oxidized state. These preparations of highly purified concentrates were made according to methods previously described (38 - 42), and had been used in studies

reported in references (46-53). The group A toxin consisted entirely of protein and had a titer of 160,000 hemolytic units (HU) per milligram of dry weight; the group C material, which was 67 % protein, had a titer of 40,000 HU per milligram.

In experiments on the perfused whole heart and the isolated atria of the guinea pig, the group A and group C preparations, although derived from organisms grown under completely different growth conditions and circumstances, behaved identically. They produced comparable physiological effects when present in the same hemolytic potencies, and, in both cases, the magnitudes of these effects correlated closely with the hemolytic activities.

Toxin stock solutions were prepared daily by dissolving the lyophilized powder in 0.15 *M* sodium phosphate buffer of pH 7.3. The toxin was activated by reduction with L-cysteine-HCl added in the same phosphate buffer to bring the final concentration of the amino acid to 10 mg/ml. The reduction reaction was allowed to proceed for 10 minutes at 30°C after which the activated stock solution was placed in an ice-bath. The direct effect of cysteine on the whole heart and isolated atria, a slight depression of rate and amplitude, was largely eliminated by diluting the stock toxin solutions at least 1:10 with buffer before testing them on the tissue.

Antitoxin. Normal pooled human gamma globulin (Squibb), a 16% solution containing known streptococcal antibodies with an

anti streptolysin O titer of 2000 Todd anti streptolysin units/ml (39, 40, 41), was furnished through the American Red Cross. Two purified plasma proteins, human serum albumin (Cutter), and bovine gamma globulin (Armour), were used for control of antitoxin.

Organic Reagents. The cholesterol[1] used to inhibit streptolysin O activity was recrystallized four times from ethanol and prepared as an aqueous suspension according to the method of Cohen, *et al.* (29). Various pharmacological agents, prepared in 1% NaCl, were used in several phases of the analysis of the mode of action of the toxin. These included: (i) acetylcholine chloride[2]; (ii) atropine sulfate[1]; (iii) eserine salicylate[1]; (iv) Na-heparin [1]; (v) l-methyl-*d*-lysergic acid butanolamide bimaleate (UML-491)[3]; (vi) serotonin creatinine sulfate[1]; and (vii) *n*-acetyl serotonin[1].

Nutrient Media for Isolated Tissues. Studies involving the isolated cardiac tissues of guinea pigs, rabbits, and rats were carried out in Chenoweth's solution (68); the bioassays on the guinea pig ileum, in Tyrode's solution. Both media were prepared daily from reagents of analytical grade in water redistilled over alkaline permanganate.

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1 Nutritional Biochemical Corp., Cleveland, Ohio.

2 Merck and Company, Inc., Rahway, New Jersey.

3 Sandoz Pharmaceuticals, Hanover, New Jersey.

Chenoweth's solution was continuously equilibrated with 95% O_2 - 5% CO_2 to give a pH of 7.4; Tyrode's, pH 7.8, was gassed with 100% O_2.

Methods.

Experimental Animals. Normal male guinea pigs, ranging in weight from 250-500 grams, were used in the bulk of these studies. Young (4-8 weeks), male, albino New Zealand rabbits, and 100-200 gram, male, white rats were also tested. Unless specifically noted, the results presented in this communication were obtained from studies on guinea pig cardiac tissues.

Preparation of Tissue. All cardiac tissues were prepared according to a common general procedure. Each animal was primed with an intraperitoneal dose (5 mg/kg) of sodium heparin, and killed 10 minutes later by a blow to the base of the skull. The thoracic cage was opened and a cannula inserted into the ascending aorta. The cannulated heart was then excised, connected to the standpipe of an Anderson perfusion apparatus[4], and perfused at 37°C with oxygenated Chenoweth's solution at a pressure head of 40 cm H_2O. After being thoroughly equilibrated and trimmed of extraneous tissue, such hearts were either left in place for studies involving the intact whole organ, or removed and appropriately dissected for experiments on the separate cardiac tissues.

In experiments dealing with the mechanical and electrical responses of isolated atrial pairs, the heart was removed from the perfusion system

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[4] Metro Industries, Long Island City, New York.

and the atria dissected as a single tissue from the ventricles. For the studies requiring individual left or right atria, the atrial pair was surgically separated so that the sino-atrial node remained intact in the right atrium. Certain experiments required the preparation of isolated ventricle strips. Such tissues were obtained from hearts treated in the foregoing manner, and prepared as described by Feigen, *et al.* (69).

Ileal tissue for the bioassays was obtained from normal guinea pigs by gently freeing it from the peritoneum and delivering it into a beaker of oxygenated Tyrode's solution at 37°C. When the entire length of gut had been transferred and the luminal contents thoroughly flushed out, it was tied off at the oral and cecal ends, severed, and stored at 5°C in oxygenated solution. After 1 hour, the tissue was warmed to 37°C, and one-third of the distal portion of the gut cut into 2-cm portions.

Procedures and Apparatus. Intact whole hearts were perfused through the coronaries on the Anderson apparatus. Streptolysin O and various pharmacological agents were added in small aliquots to the standpipe above the heart and flushed through the tissue with normal Chenoweth's. In certain experiments, atropine or eserine, dissolved in Chenoweth's, was continuously perfused through the heart. The perfusion rate was measured by carefully timed collections of perfusate in graduated, conical centrifuge tubes. Contractions were recorded on an Offner Dynograph Type 506 by means of a transducer attached through a thread to the apex of the ventricles.

Simultaneous recordings of the electrophysiological and mechanical activity of atrial and ventricular tissues were made using equipment and methods adequately described previously (70, 71). Briefly, the tissue was mounted horizontally in a temperature-controlled bath. Intracellular potentials, measured with standard glass micro electrodes, and mechanical activity, monitored by a moveable-anode transducer, were displayed on a twin-beam oscilloscope and photographed by an oscilloscope camera. Atrial preparations were stimulated with a biphasic 1.0 msec pulse, 10 volts above threshold, at a rate approximately 10 beats per minute above the natural rate. Ventricle strips were similarly driven at a rate of 180 beats per minute. All preparations were equilibrated with O_2/CO_2 and maintained at 37°C.

The mechanical activity of spontaneously beating, isolated atria was studied on tissues mounted vertically in water-jacketed muscle baths having a volume of 3.5 ml. Three of these baths were arranged with their water jackets connected in series with a pump-thermostat so that multiple tissues could be studied simultaneously. The recording system consisted of a Statham G-7A transducer, G-18 control box, and the Offner Dynograph. These preparations were maintained at 30°C in order to increase the amplitude of contraction, decrease the rate, and prolong the period of stability. At this temperature the rate was 53%, and contraction 210%, of that at 37°C, and preparations were stable for at least 20 hours.

The bioassays for acetylcholine were performed on ileal strips each of which was secured to a glass L-rod and mounted in a water-jacketed 3.5 ml muscle bath in such a way that the free end of the gut strip could be connected by a thread to a lever-arm writing on a smoked drum.

RESULTS

The Action of Streptolysin O on the Whole Heart.

The addition of a small dose (36 HU) of active toxin to the solution perfusing an isolated, whole guinea pig heart resulted in a gradual, monotonic, decrease in the amplitude of contraction (Figure 1A). As the dose of streptolysin O was increased, however, the character of this response was altered by the appearance of a rapid, transient decrease in the rate and amplitude, superimposed on the gradual decline (Figure 1B-1D). Larger doses produced an immediate, reversible, cardiac arrest (characterized by both atrial and ventricular standstill), followed by partial recovery of the rate and amplitude before the long-term effect became dominant (1D). This biphasic nature of the response is illustrated graphically in Figure 2, which describes the responses of three hearts to different doses of toxin. At higher doses, the initial phase was a rapid, transient, depression of contraction magnitude, while terminally, a gradual decline in amplitude occurred, leading to ventricular standstill. It is clear that the terminal phase was significantly more sensitive to streptolysin O than was the initial phase.

Figure 1. The responses of four isolated hearts recorded at various times following challenge with different doses of the group A toxin.(37°C).

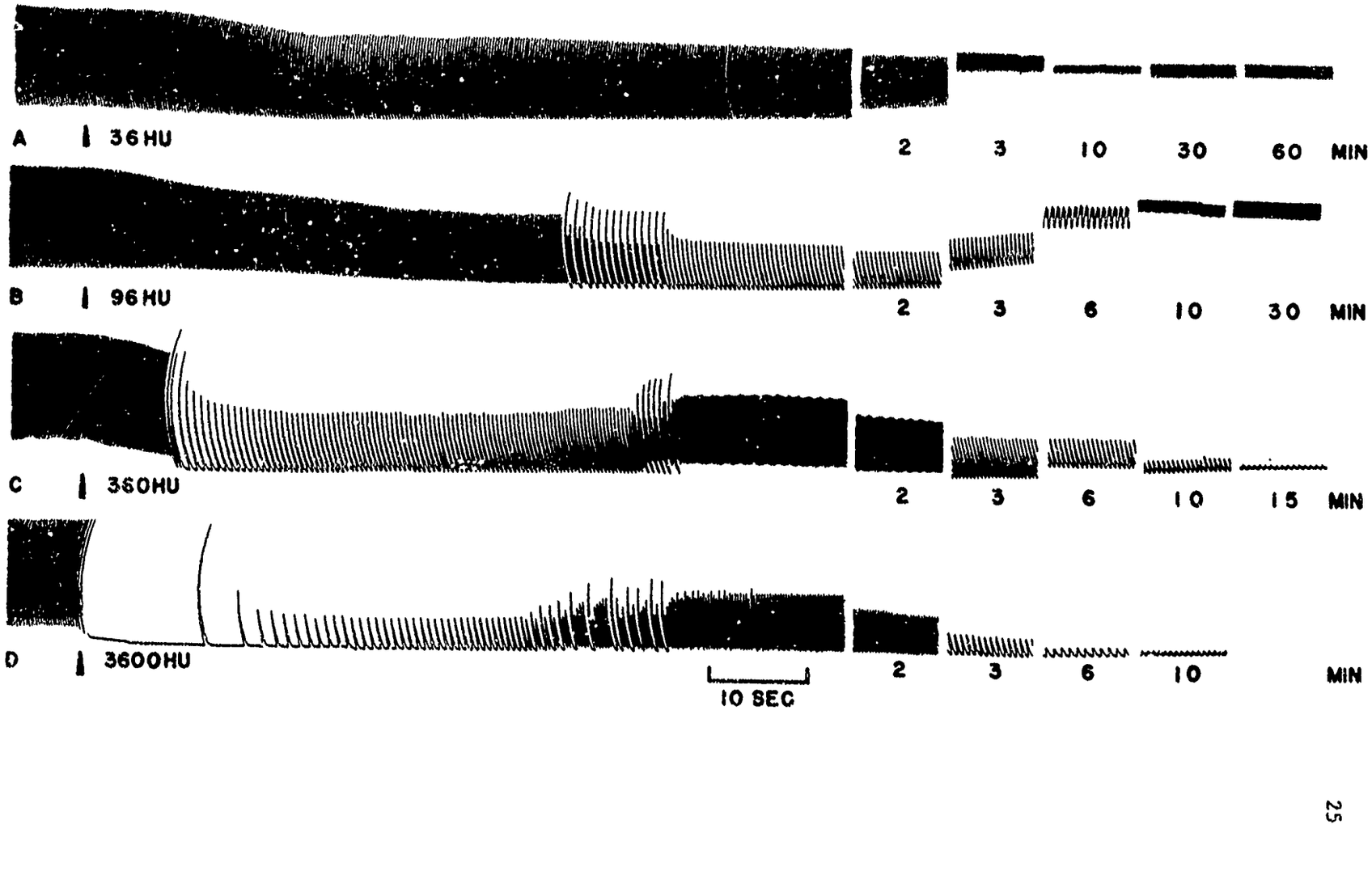
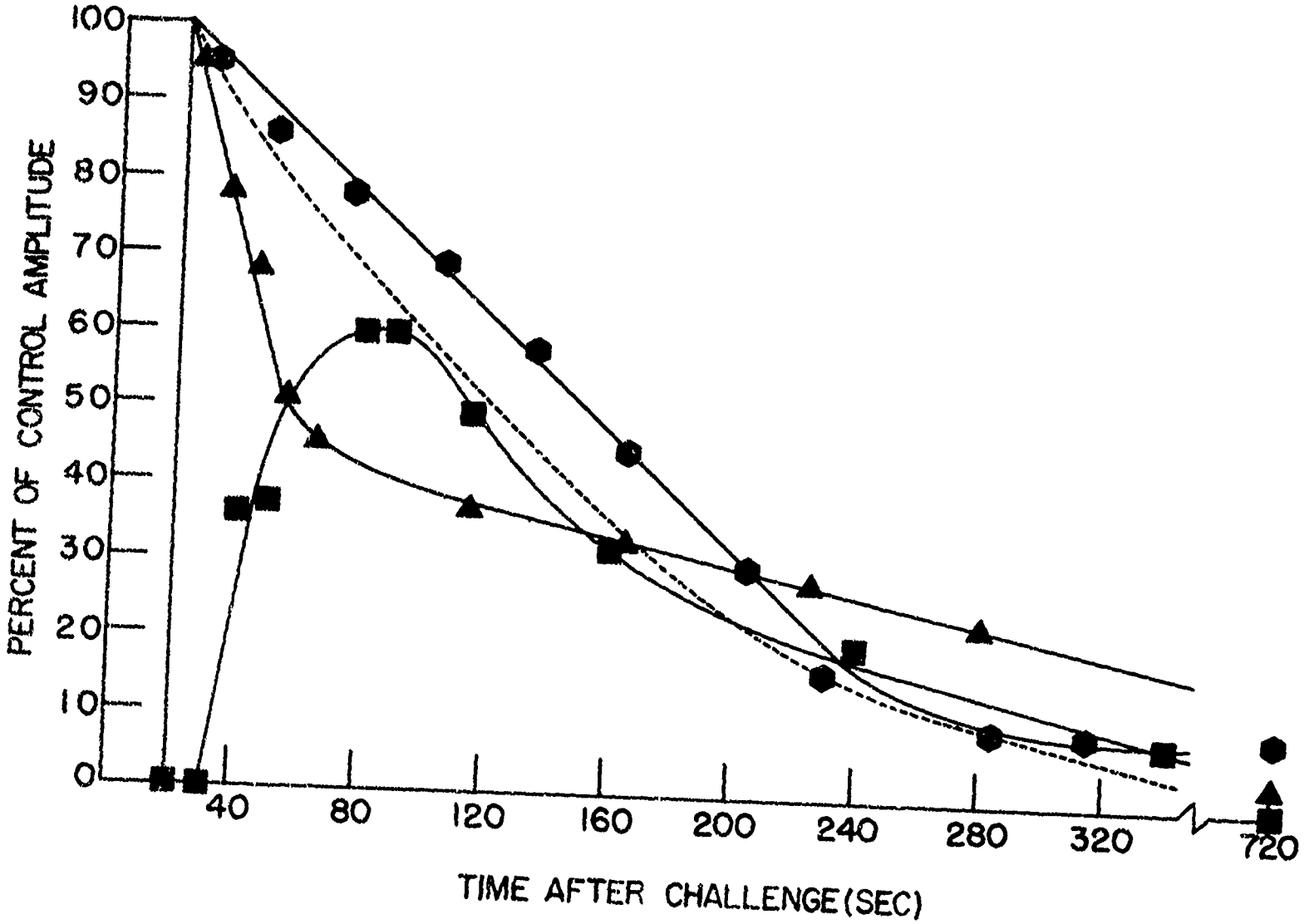

A
36HU
2
3
10
30
60
MIN
B
96HU
2
3
6
10
30
MIN
C
360HU
2
3
6
10
15
MIN
D
3600HU
10 SEC
2
3
6
10
MIN

Figure 2. The effect of various doses of group A streptolysin O on the amplitude of contraction of the isolated heart (⬢——⬢ 36 HU, ▲——▲ 264 HU, ■——■ 528 HU); comparison with the effect of stopping the flow of perfusion fluid (--------). All at 37°C.


100
90
80
70
60
50
40
30
20
10
0
PERCENT OF CONTROL AMPLITUDE
40
80
120
160
200
240
280
320
720
TIME AFTER CHALLENGE (SEC)

Along with these mechanical changes, streptolysin O caused marked alterations in the rate of cardiac perfusion, in agreement with the findings of Kellner, *et al.* (51). Doses of toxin greater than about 120 units produced an immediate and permanent cessation of flow, whereas lower doses induced a rapid, but temporary, decrease in perfusion rate. In these cases, the flow gradually recovered to an extent inversely dependent upon the dose of toxin. Hearts subjected to doses of toxin sufficient to cause permanent arrest of flow always stopped beating completely within 6 - 15 minutes after challenge. In these hearts, it was not possible to separate the consequences of the direct action of streptolysin O on the contraction processes from the deleterious effects of the decreased perfusion. These effects are shown by the dashed curve of Figure 2, which represents the decrease in amplitude subsequent to clamping off the flow of perfusion fluid to a normal heart. The resulting decline in force of contraction closely parallels that seen with the higher doses of toxin. Hearts subjected to doses of toxin which caused only a transient depression of flow did not stop beating but continued to contract indefinitely at some small fraction of their control amplitude (see Figures 1A and 1B). In some cases, flow returned to as much as 95% of the control level, although the amplitude of contraction never recovered to more than about 15% of its pre-challenge level.

Upon close observation of hearts reacting to large doses of toxin, it could be seen that the depression, or even the complete cessation of

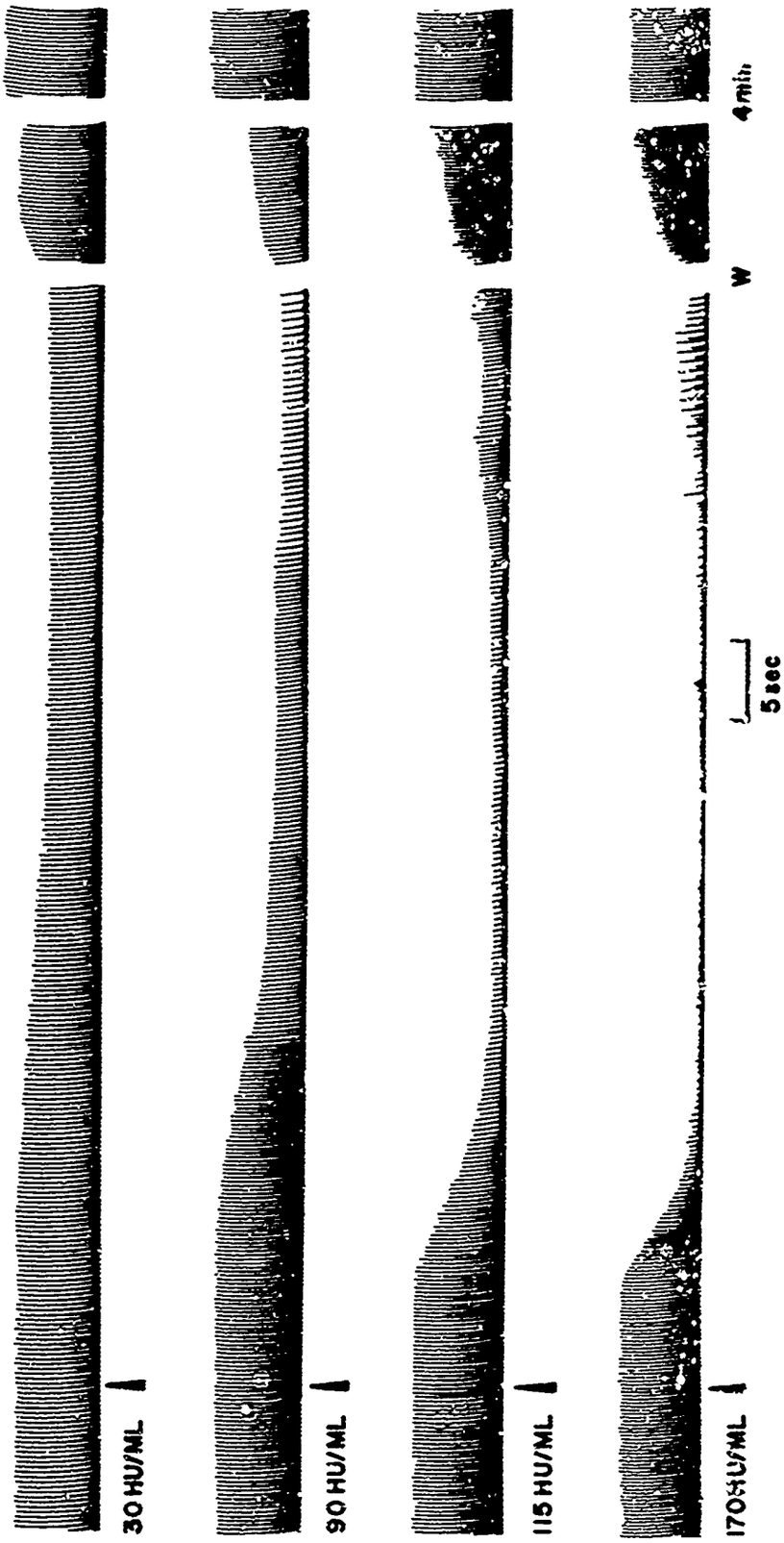
activity during the initial phase was common to both atria and ventricles. Similarly, the partial spontaneous recovery of rate and amplitude, characteristic of this phase, was shared by both tissues. At the height of the recovery, however, the *ventricles* began to lose contractile force. They became partially, and then completely, dissociated from sino-atrial rhythm, and began to beat irregularly. The *atria*, on the other hand, continued to recover and, at ventricular standstill, still beat strongly. If such intoxicated hearts were then dissected and the atria and ventricles separately tested, it was found that the atria beat spontaneously and vigorously at their control rates for many hours, and that electrically driven ventricle strips were indistinguishable in their performance from strips obtained from normal hearts.

These preliminary observations suggested that the localization of the physiological disturbance, and the analysis of the mode of action of the toxin might be facilitated by first studying the behavior of the individual cardiac tissues rather than the whole organ.

The Action of Streptolysin O on the Isolated Atria.

Mechanical Properties. The characteristic response of the spontaneously beating, isolated atria to activated streptolysin O consisted of a lag period followed by a transient decrease in rate and amplitude of contraction. The nature of this response is illustrated in Figure 3, which presents the recorded reactions of four atrial preparations to different concentrations of toxin. The time between administration of the

Figure 3. The recorded responses of four preparations of isolated atria to different concentrations of streptolysin O. Washing out the bath with fresh medium (W) accelerates the recovery. (30°C).


30 HU/ML
90 HU/ML
115 HU/ML
170 HU/ML
W
4 min
5 sec

test dose and the onset of the response decreased as the streptolysin O concentration was increased. The dose-dependence of this inverse relationship is summarized in Figure 4. The more complex dose-dependence of the transient changes in atrial rate and amplitude produced by the toxin is shown quantitatively by the sigmoidal functions of Figure 5. The toxin clearly exerted a much more profound depression on the force of contraction than on the frequency.

The recovery of the atria from the effects of the toxin was characterized by a transient potentiation in the amplitude and rate of contraction above the control values. Although no meaningful correlation was found between the extent of this potentiation and the magnitude of the transient depression, a good correlation does exist between the time required for recovery and the severity of the response. The recovery time was shortened if the solution was washed out at the first sign of spontaneous recovery.

In a series of tests on rabbit and rat atria, it was observed that, although these tissues appeared to be less sensitive to the actions of streptolysin O than guinea pig atria, they nevertheless exhibited the same characteristic response to the toxin.

The repeated challenge of guinea pig atria with a constant dose of streptolysin O produced a gradual decline in responsiveness. This tachyphylaxis is exhibited in the tracings of Figure 6 taken from an atrial

Figure 4. The effect of increasing the streptolysin O concentration on the lag period of response in isolated atria. (30°C).

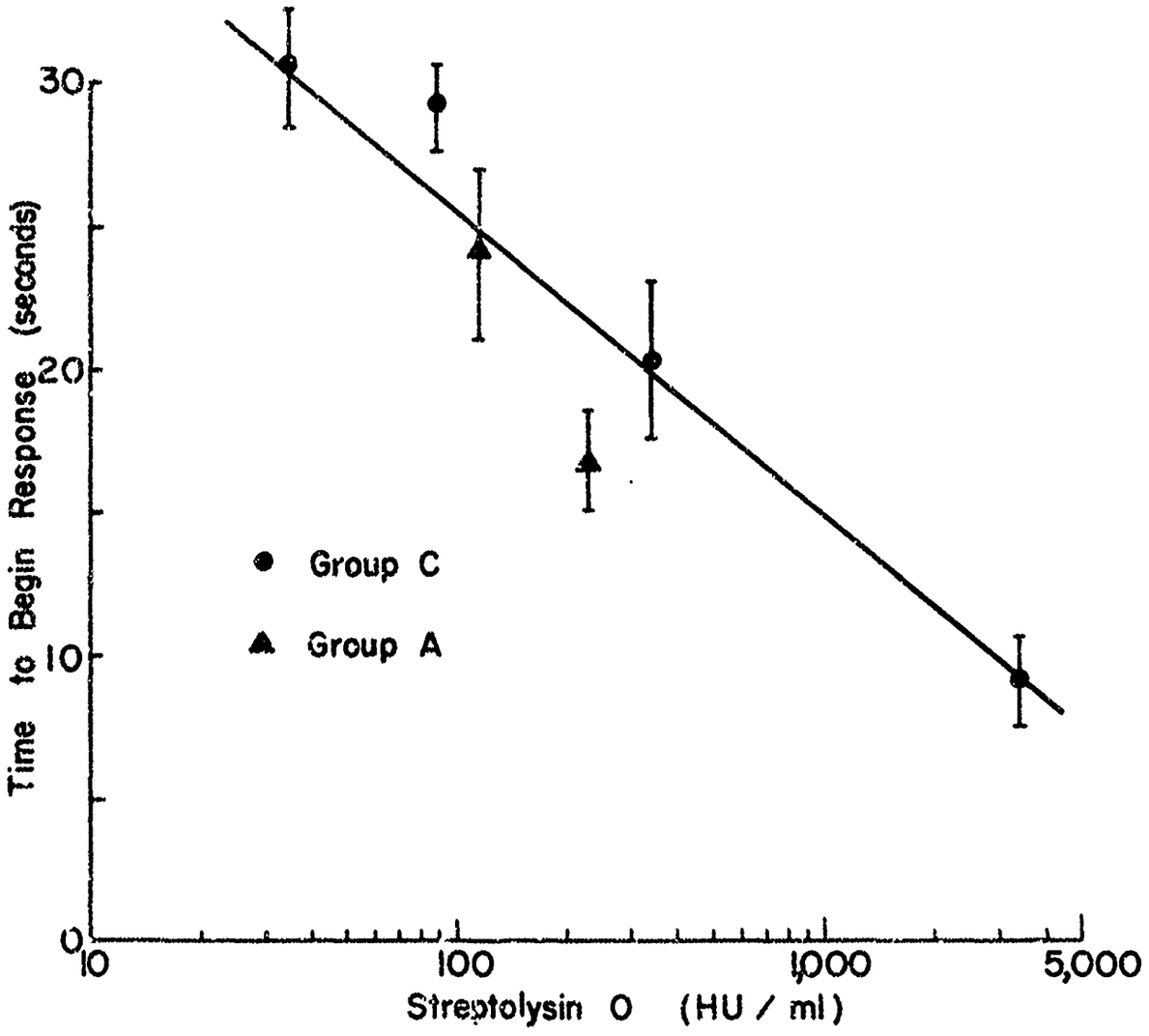

30
20
10
0
Time to Begin Response (seconds)
Group C
Group A
10
100
1,000
5,000
Streptolysin O (HU / ml)

Figure 5. The dose-response relationships for streptolysin C on the isolated atria: the effect of toxin concentration on the rate and amplitude of contraction at 30°C.

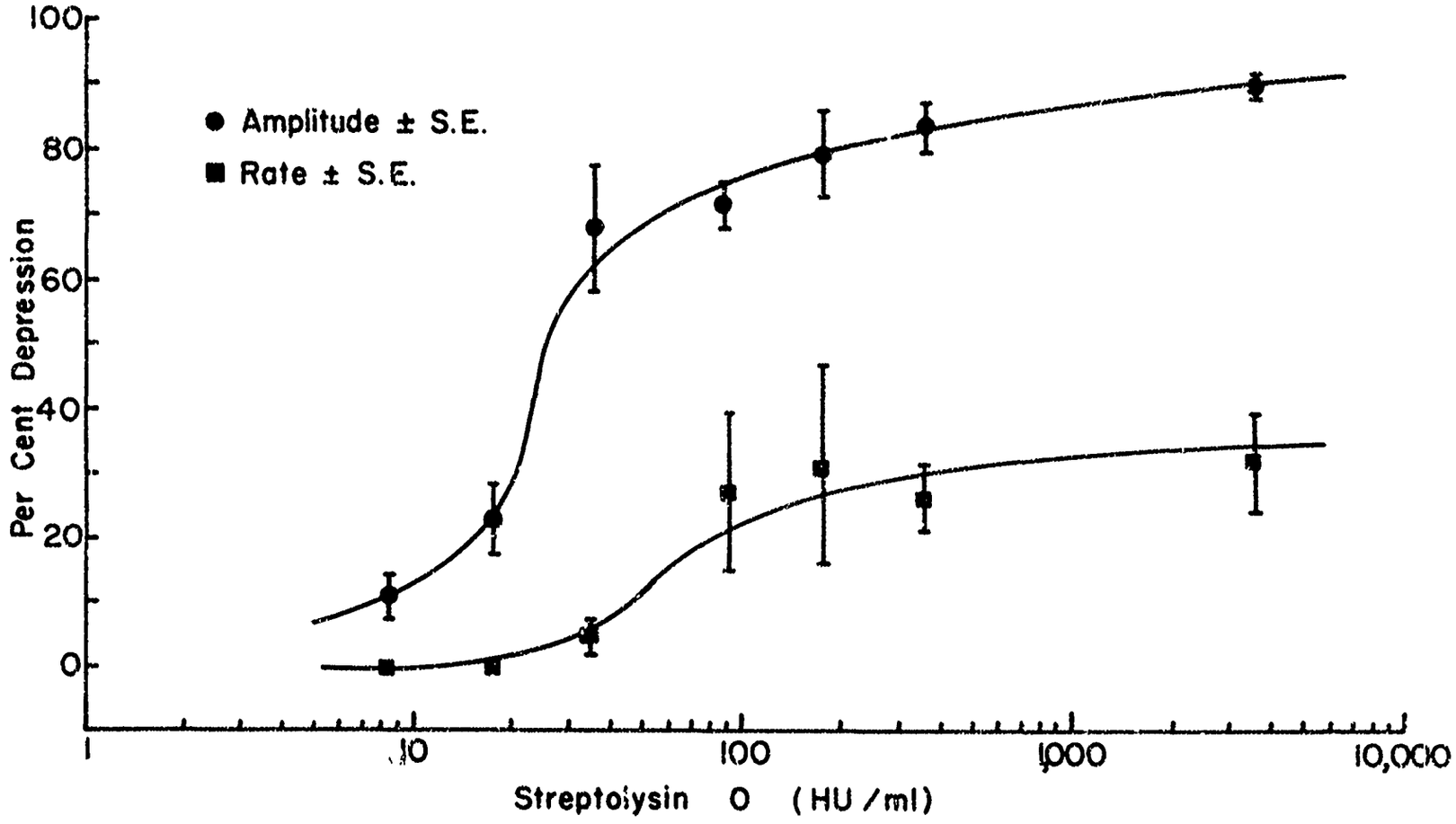
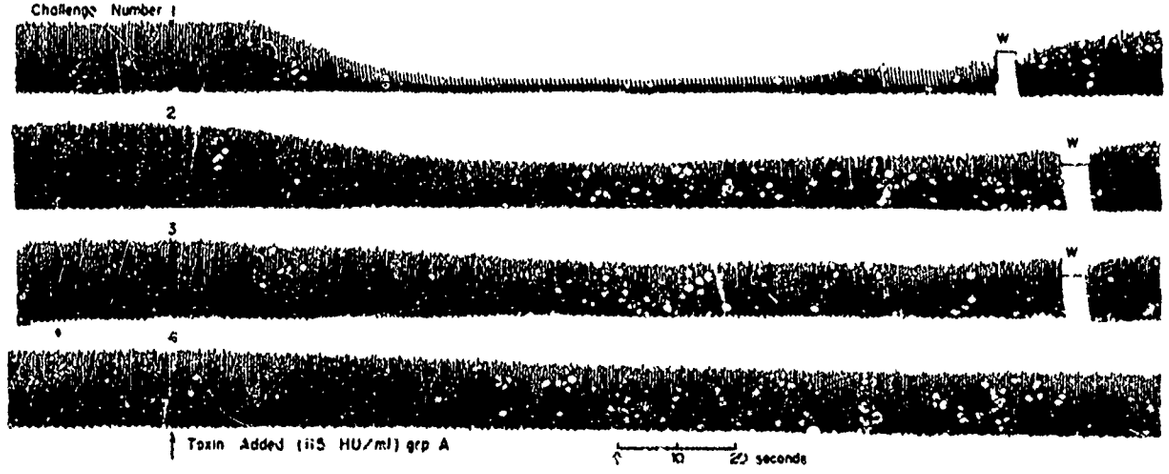

100
80
60
40
20
0
Per Cent Depression
● Amplitude ± S.E.
■ Rate ± S.E.
1
10
100
1000
10,000
Streptolysin O (HU/ml)

Figure 6. Tachyphylaxis to streptolysin O in the isolated atria: the effect of four successive challenges with the same dose of toxin on the magnitude of response at 30°C.


Challenge Number 1
W
2
W
3
W
4
Toxin Added (115 HU/ml) grp A
10
20 seconds

preparation subjected four times to challenge with 115 HU/ml. Each successive challenge was characterized by a prolongation of the lag period, and a reduction in the depth of response. Figure 7 shows that the rate of decline of responsiveness was dose-dependent; semilogarithmic plots of these functions are linear. The data in Table I show that there was no recovery of responsiveness with time. A single atrial preparation, challenged six times with 158 HU/ml of streptolysin O, was then maintained for 250 minutes in frequently renewed medium before the seventh challenge. In spite of this considerable delay, the response to the seventh challenge was still less than that observed for the sixth challenge.

Electrical Properties. Intracellular potentials, recorded from electrically driven atria, showed that the electrophysiological consequence of their exposure to streptolysin O was an increase in the rate of repolarization of the action potential. Figure 8 presents oscillographic records of the intracellular potential and mechanical contraction before (trace A), and 45 seconds after (trace B), challenge with 140 HU/ml of reduced toxin. Quantitative expression of this effect is provided by the reconstructed action potentials drawn in Figure 9. Exposure to 720 HU/ml of active toxin for 45 seconds increased the initial slope of the repolarization phase of the potential from the control rate of 0.73 V/sec (trace A) to 2.4 V/sec (trace B) without measurably affecting the depolarization phase. In these studies, the mechanical and electrical changes began within 20 to 60 seconds following the addition of the toxin; but, whereas the action

Figure 7. The effect of toxin dose on the rate of tachyphylaxis to streptolysin O in isolated atria. Comparison with the response to an iterated dose of acetylcholine. (30°C).

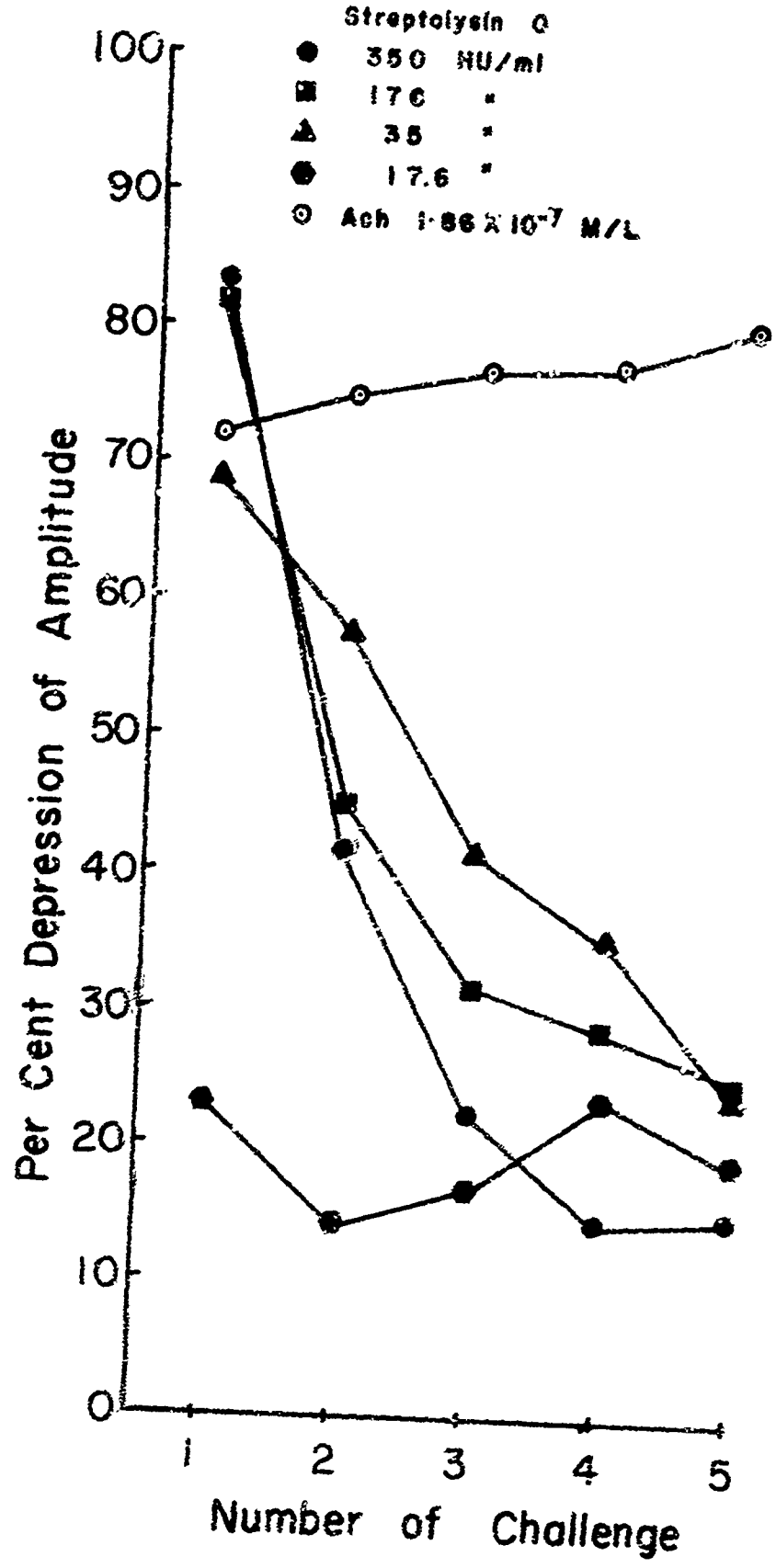

Streptolysin O
350 HU/ml
176 "
35 "
17.6 "
Ach 1·86 X 10⁻⁷ M/L
Per Cent Depression of Amplitude
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Number of Challenge

TABLE I

The Effect of Time on Atrial Tachyphylaxis to Streptolysin O*

Challenge No.	Time After 1st Challenge (min)	Per Cent Depression of Contraction amplitude
1	0	77.2
2	10	48.9
3	20	31.1
4	30	29.2
5	40	22.6
6	50	22.0
7	300	21.6

* All challenges with 158 HU/ml

Figure 8. Oscillographic recordings of the contraction (upper trace in each frame) and intracellular action potential of the isolated atria before, and 45 seconds after, challenge with streptolysin O at 37°C.

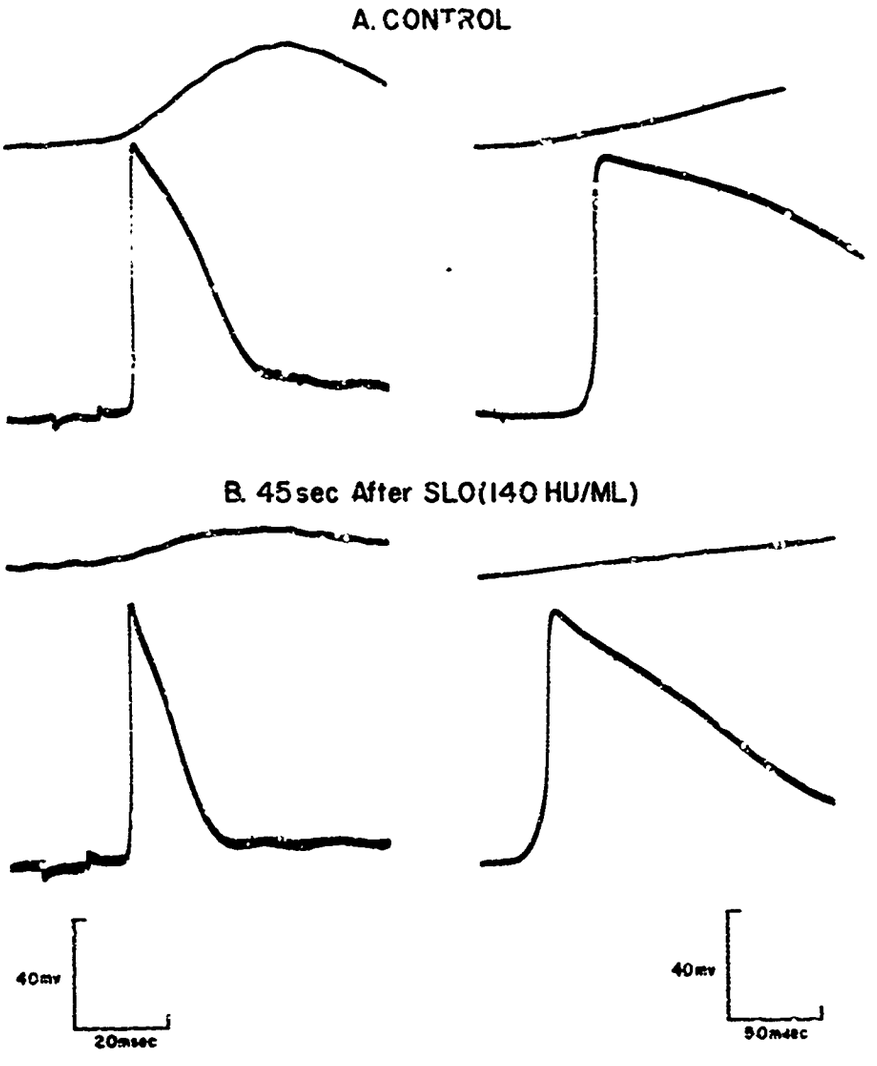
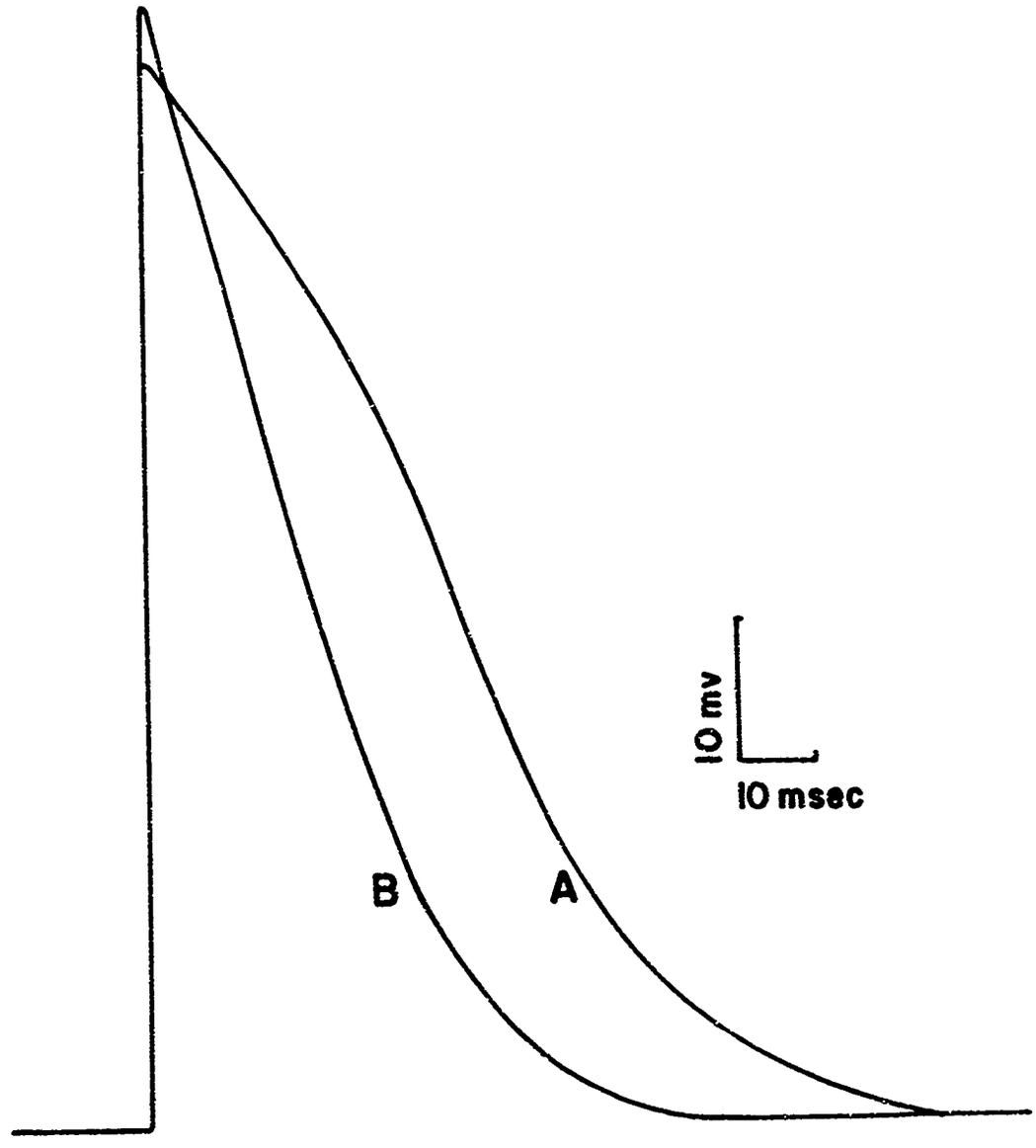

A. CONTROL
B. 45sec After SLO(140 HU/ML)
40mv
20msec
40mv
50msec

Figure 9. The effect of streptolysin O on the rate of repolarization of the atrial action potential at 37°C. Reconstructed potentials obtained from 9 cells prior to challenge (A), and 4 cells after challenge with 720 HU/ml of active toxin (B).


10 mv
10 msec
B
A

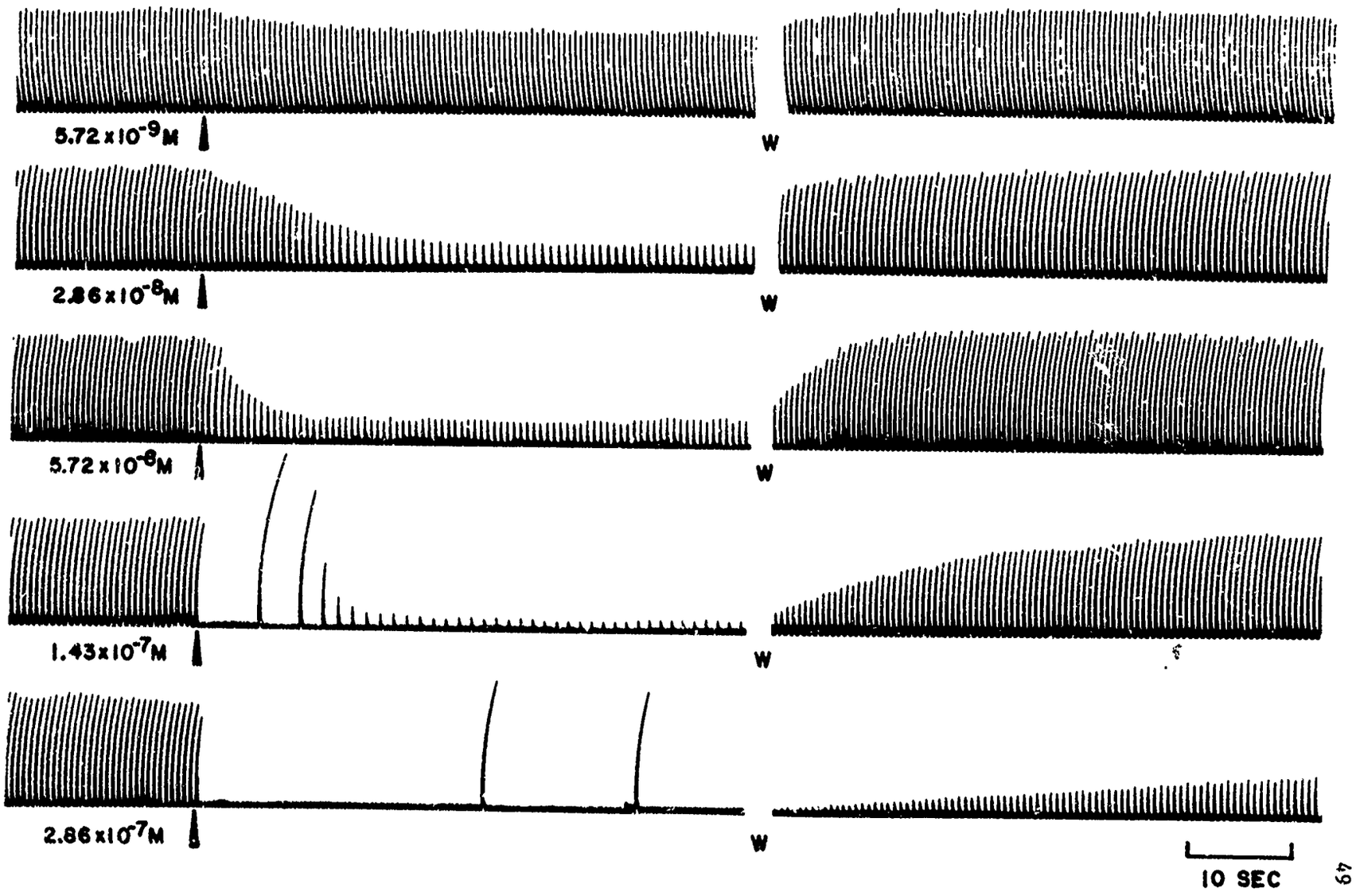
potential returned to its control configuration in 2 to 3 minutes, the contraction did not fully recover until 8 to 12 minutes post-challenge. Additional electrophysiological studies made on separated atria showed that there was no difference between the right and left atria with respect to their electrical or mechanical reactions to streptolysin O.

Evidence for Release of Acetylcholine by Toxin-Treated Atria.

From a careful analysis of the electrocardiographic records of reference 46, as well as from the transiently decreased frequency and amplitude of contraction, and increased rate of repolarization observed in the present experiments, there was sufficient circumstantial evidence to consider the possibility that the reversible phase of streptolysin cardiotoxicity was due to the release of acetylcholine. In order to test this idea, it had to be shown by quantitative experiments that both acetylcholine and streptolysin O, given in comparably effective doses, produced identical electrical and mechanical changes; that both effects could be blocked by atropine and potentiated by eserine; and, finally, that the release of acetylcholine from atria challenged with streptolysin O could be demonstrated unequivocally.

Comparison of the Effects of Acetylcholine and Streptolysin O on The Isolated Atria. The responses of isolated atrial preparations to five concentrations of acetylcholine are shown in the recordings of Figure 10. They consist of a dose-dependent decrease in rate and amplitude

Figure 10. The recorded responses of isolated atria to five concentrations of acetylcholine at 30°C. The effect of washing out (W) the bath on the rate of recovery.


5.72 x 10⁻⁹M
W
2.86 x 10⁻⁸M
W
5.72 x 10⁻⁸M
W
1.43 x 10⁻⁷M
W
2.86 x 10⁻⁷M
W
10 SEC

of contraction, followed by spontaneous recovery characterized by a transient potentiation. This pattern of response is identical to that observed in tissues challenged with streptolysin O (Figure 3). However, there is a fundamental difference between the effects of the two agents which was revealed only by repeated testing. Unlike streptolysin O, which depressed the amplitude of contraction less with each repeated challenge, the reaction to iterated doses of acetylcholine was rather one of slight potentiation (top curve Figure 7). The dose-response relationships for the depression of rate and amplitude of individual atrial preparations by selected doses of acetylcholine are seen in the right-hand sigmoidal functions of Figure 11. As with streptolysin O, the amplitude was considerably more sensitive to the effects of acetylcholine than was the rate.

Electrophysiologically, the treatment of electrically driven, isolated atria with acetylcholine resulted in a dose-dependent increase in the rate of repolarization of the intracellular potential as seen in Figure 12B. These effects were quite similar to those produced by streptolysin O (Figures 8, 9).

Effects of Pharmacological Agents. If the release of acetylcholine is a significant component of the atrial response to the toxin, the reaction to streptolysin O ought to be blocked by atropine and potentiated by an anticholinesterase such as eserine. The ability of atropine to block

Figure 11. The dose-response relationships for acetylcholine on isolated atria: the effect of concentration on the rate (R) and amplitude (A) of contraction in the normal and eserinized preparation at 30°C.

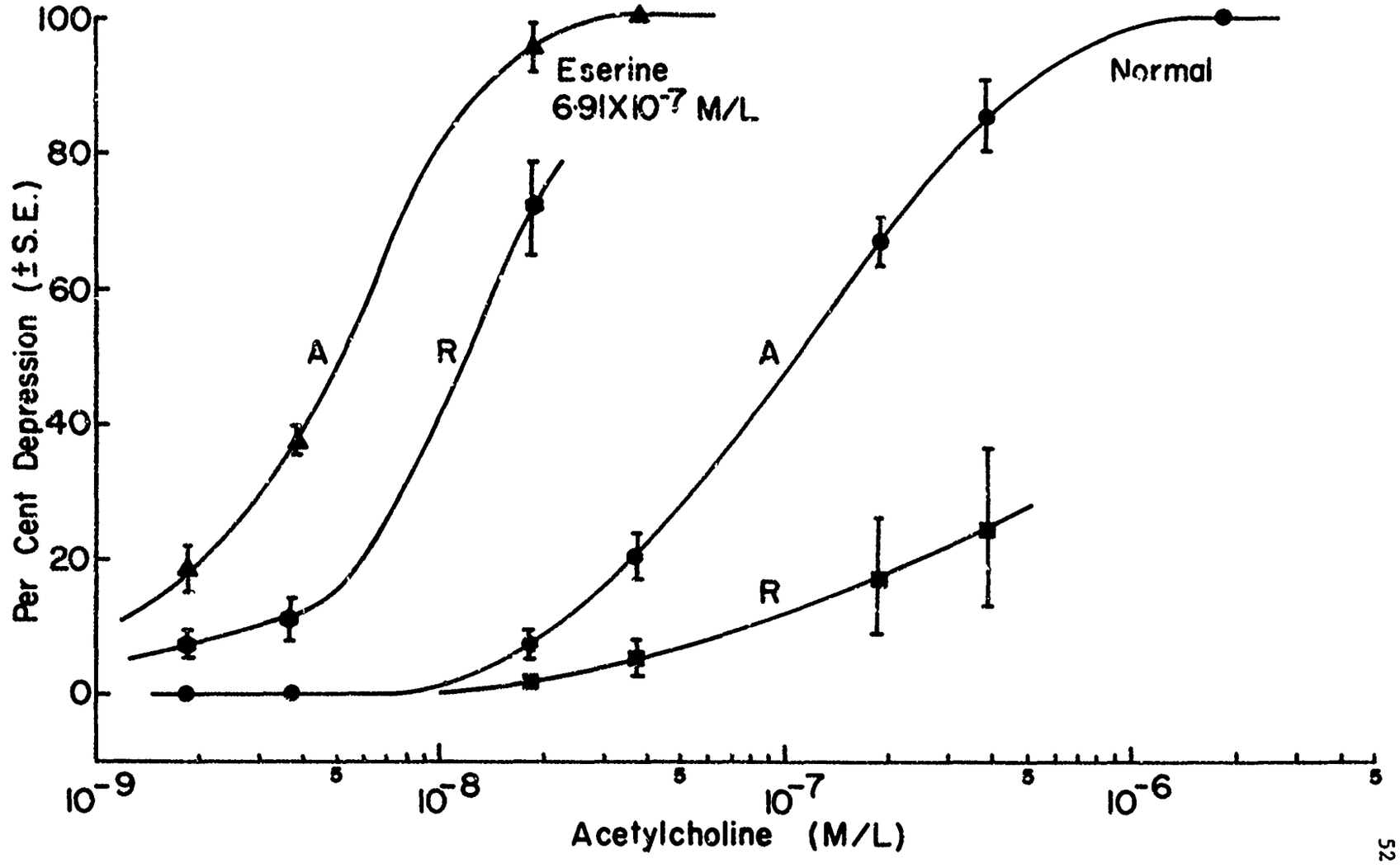
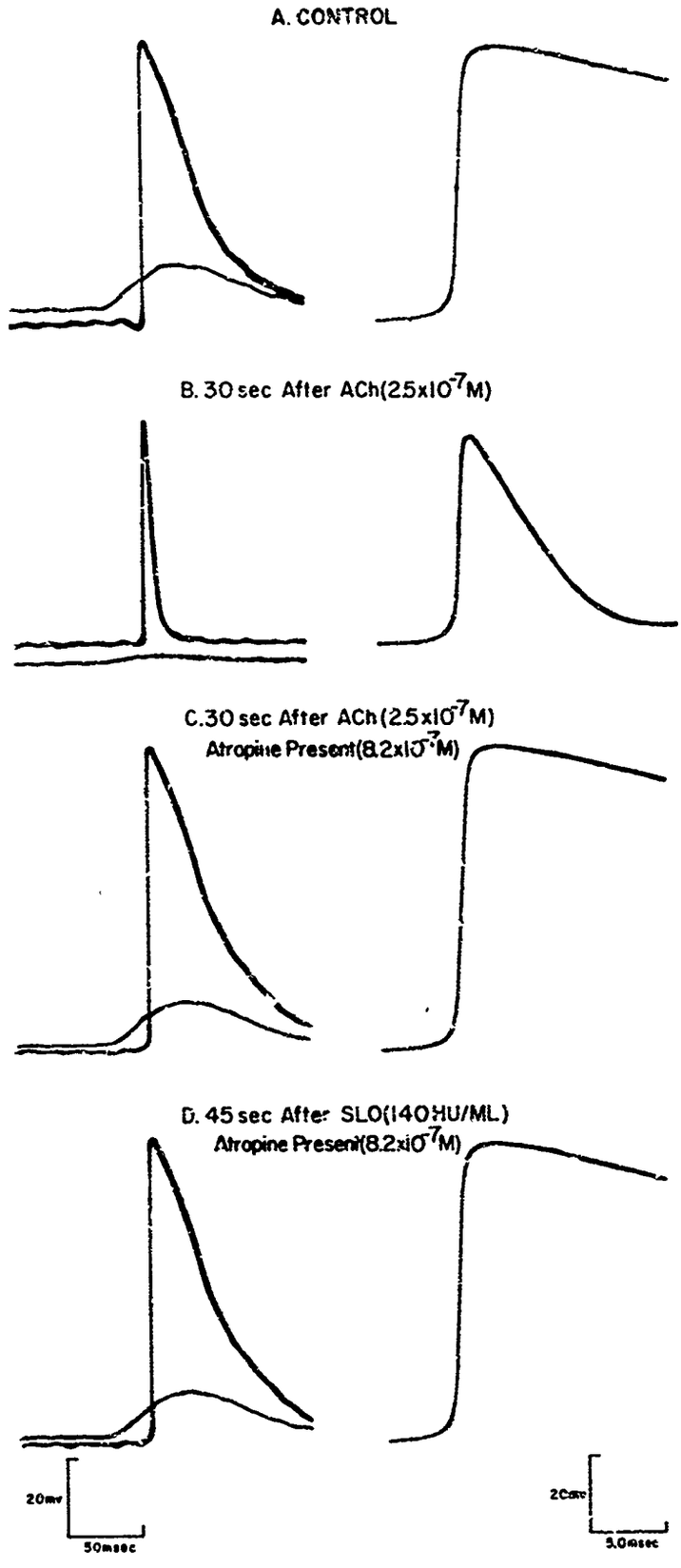

100
80
60
40
20
0
Per Cent Depression (± S.E.)
Eserine
6·91X10⁻⁷ M/L
Normal
A
R
A
R
10⁻⁹
5
10⁻⁸
5
10⁻⁷
5
10⁻⁶
5
Acetylcholine (M/L)

Figure 12. The contraction and intracellular potential of an isolated atrial preparation before (A), and 30 seconds after (B), challenge with acetylcholine. Atropine, at a concentration sufficient to block this dose (C), completely eliminates the effects of 140 HU/ml of streptolysin O (D; compare with Figure 8B).

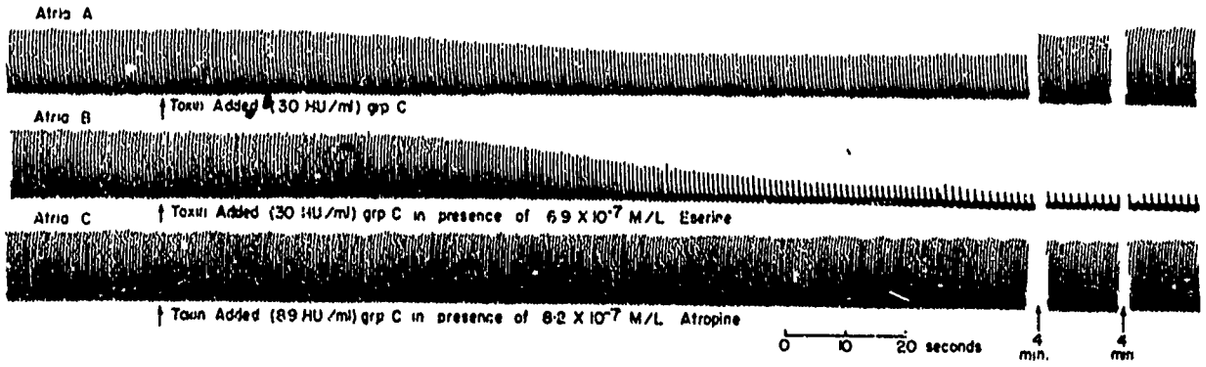

A. CONTROL
B. 30 sec After ACh(2.5x10^{-7}M)
C. 30 sec After ACh(2.5x10^{-7}M)
Atropine Present(8.2x10^{-7}M)
D. 45 sec After SLO(140 HU/ML)
Atropine Present(8.2x10^{-7}M)
20mv
50msec
2Cmv
5.0msec

the electrophysiological manifestations of streptolysin O action on the isolated atria is demonstrated in Figure 12. The presence of atropine in a concentration sufficient to eliminate the striking increase in the rate of repolarization produced by 2.5×10^{-7} *M* acetylcholine (12B), completely blocked the effects of streptolysin O on the repolarization rate (12D; compare with Figure 8B).

The actions of atropine and eserine on the mechanical changes produced by streptolysin O are shown in Figure 13. The slight depression of amplitude caused by 30 HU/ml of toxin (Atria A) turned into a profound and prolonged depression of amplitude and frequency when this test dose of toxin was given to an atrial preparation surviving in the presence of 6.9×10^{-7} *M* eserine (Atria B). On the other hand, the effects of a trebled concentration of toxin were seen to be blocked completely in the presence of 8.2×10^{-7} *M* atropine (Atria C).

The ability of eserine to increase the sensitivity of the atria to the chronotropic effects of streptolysin O, illustrated in Figure 13B, supports the inference that the effects of the toxin on this tissue are mediated by the release of acetylcholine. The dose-response curves of normal and eserinized atria presented in Figure 11, show that 6.9×10^{-7} *M* eserine not only lowered the effective dose range of acetylcholine by approximately one order of magnitude, but also significantly steepened the slope of the rate depression curve.

Figure 13. The response of an isolated atrial preparation to 30 HU/ml of streptolysin O (Atria A). Modification by eserine of the response of another atrial pair challenged simultaneously with the same toxin concentration (Atria B), and by atropine of the response of a third preparation to a three-fold increase in toxin dose (Atria C). 30°C.


Atria A
Atria B
† Toxin Added (30 HU/ml) grp C
Atria C
† Toxin Added (30 HU/ml) grp C in presence of 6.9 X 10⁻⁷ M/L Eserine
† Toxin Added (89 HU/ml) grp C in presence of 8.2 X 10⁻⁷ M/L Atropine
0 10 20 seconds
4 min.
4 min

Detection and Estimation of Acetylcholine Released from Atria Exposed to Streptolysin O. Although circumstantial, the foregoing evidence was entirely consistent with the hypothesis that the toxin preparations released acetylcholine from atrial tissues. However, initial attempts to demonstrate *de facto* release from single, or even multiple (three) atrial preparations by testing the bath or perfusion fluids obtained during the course of the various experimental procedures were unsuccessful, presumably because of the presence of powerful acetylcholinesterases and the high degree of dilution to which any net release inevitably was subjected.

A test of the potency of atrial acetylcholinesterases was made by determining the rate of inactivation of exogenous acetylcholine. Four guinea pig atria, prepared in the usual way, were assembled into a bundle and immersed in 10 ml of gassed Chenoweth's solution containing 1.08×10^{-3} μmoles of acetylcholine chloride. The reaction was allowed to proceed at 37°C for 12 minutes during which 0.1 ml samples were drawn at the times indicated in the protocol given in Table II. The concentrations of residual acetylcholine at various intervals were measured by bioassay on the guinea pig's ileum with reference to standardized dose-response curves to acetylcholine. The results given in Table II show a linear reduction in concentration over the first 12 minutes. When these data were corrected for the dry weight of the tissue (98.5 mg) the initial rate of destruction at 37°C was found to be 3.4×10^{-4} μmoles acetylcholine/gram dry atrium/minute.

TABLE II

Destruction of Exogenous Acetylcholine[1] by Isolated Atria[2]

Time (min)	ACh Remaining in solution[3] (μ moles x 10^{-4})	% of Initial ACh Remaining in Solution
0	10.8	100
1	10.8	100
4	9.5	88
6	9.1	84
8	8.2	76
12	7.1	66

[1] Acetylcholine Chloride (Merck) = ACh

[2] 4 atria (dry weight = 95.8 mg) handled as single tissue

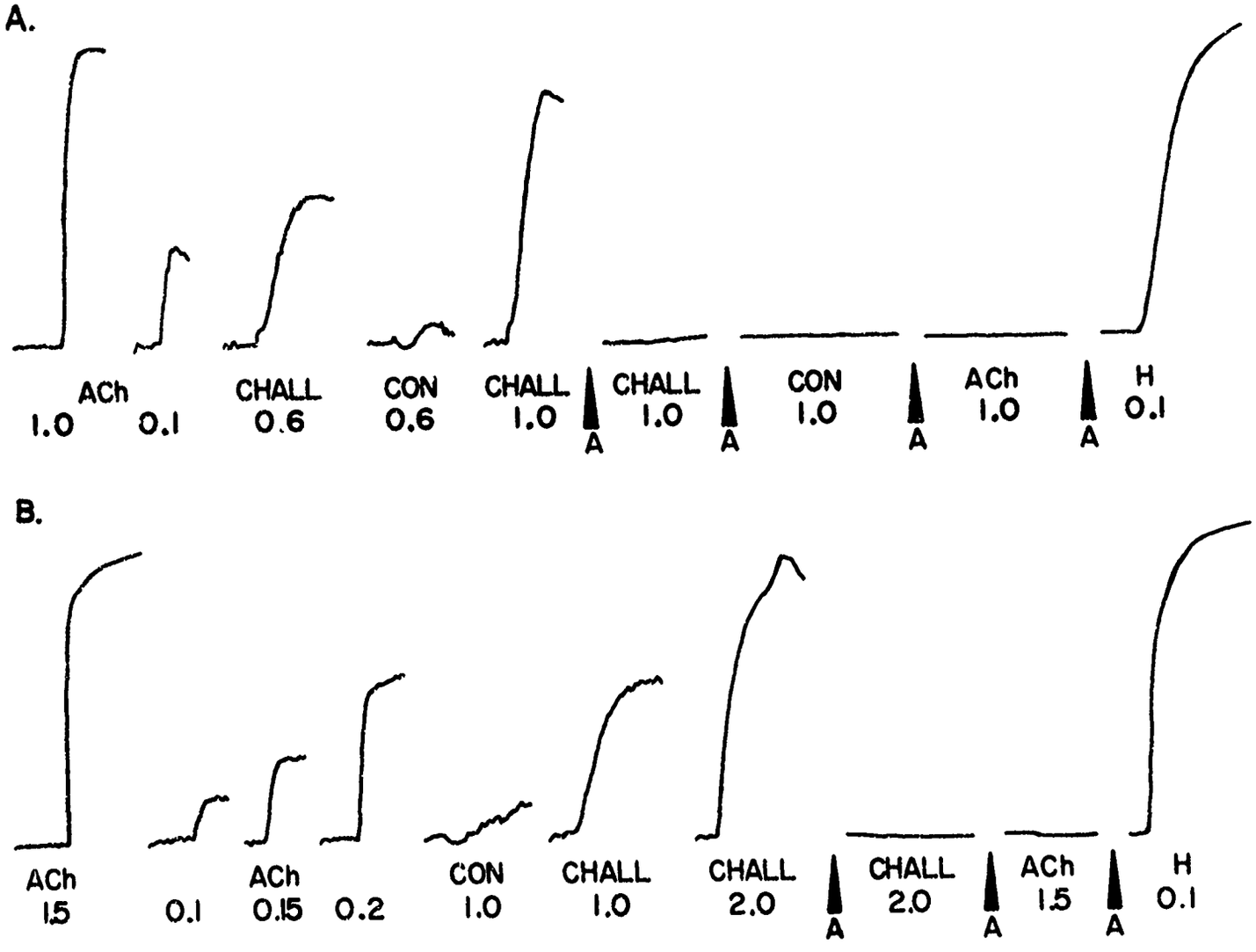
[3] Bioassay on the guinea pig ileum standardized with Acetylcholine.

From these results, it was evident that measurable amounts of acetylcholine could be obtained only if a large quantity of atrial tissue were challenged in the presence of eserine, and if samples of reaction fluid were taken at a very early stage in the reaction; furthermore, very high concentrations of toxin were used and the reaction temperature was lowered from 37°C to 25°C.

Accordingly, ten pairs of atria were assembled into a bundle and immersed into a 6.0×10^{-7} *M* solution of eserine made up in Chenoweth's. After an equilibration period of 3 minutes the tissue was transferred to a flask containing streptolysin O (838 HU/ml) made up in eserinized Chenoweth's medium. At the same time, for control purposes, an appropriate quantity of toxin was added to the original equilibration flask to bring the toxin concentration to the same strength as that present in the challenge solution. After 3 minutes of reaction the tissue was removed and the solutions from both flasks transferred into 15 ml centrifuge cones, heated to 56°C for 10 minutes to inactivate the toxin, chilled, and centrifuged at 5°C for 15 minutes. The tissue was placed in a tared weighing bottle and dried at 105°C to a constant weight; the supernates were bioassayed for acetylcholine on the guinea pig's ileum, as shown in Figure 14A.

Activity was present in both the control and the challenge fluids, suggesting that a certain amount of spontaneously released material had accumulated in the eserinized medium. Challenge with toxin clearly increased the concentration of this material, since the addition of 0.6 ml

Figure 14. Biological identification of acetylcholine released by streptolysin O-treated atria. Bioassay on the guinea pig ileum of solutions bathing challenged atria (CHALL), and control incubation solutions (CON); comparison with acetylcholine (ACh) standards. Numbers represent milliliters of sample added to the bath (final volume, 3.5 ml). Acetylcholine standard, 1×10^{-6} *M*; histamine (H) standard, 1×10^{-5} *M*; atropine (A) added to a final concentration of 7.5×10^{-5} *M*.


A.
ACh
1.0
0.1
CHALL
0.6
CON
0.6
CHALL
1.0
A
CHALL
1.0
A
CON
1.0
A
ACh
1.0
A
H
0.1
B.
ACh
1.5
0.1
ACh
0.15
0.2
CON
1.0
CHALL
1.0
CHALL
2.0
A
CHALL
2.0
A
ACh
1.5
A
H
0.1

of the challenge solution produced a contraction that was 2.5 times greater than that given by an equal volume of the control. Repetition of the test in the presence of 7.5×10^{-5} *M* atropine showed that the response to the "maximal" doses of both test and control solutions were blocked by the drug, whereas the tissue was still responsive to histamine under these conditions.

Substantially the same results were obtained in a second release experiment in which 12 atrial pairs were challenged with twice the concentration of toxin used earlier, but for half the time. The bioassay record (Figure 14B) permits the construction of a standardization curve to acetylcholine and thus to calculate the concentrations (acetylcholine equivalents) of the active atropine-sensitive material liberated. On this basis, it was computed that the control solution contained 9.8×10^{-4} µmoles of spontaneously released acetylcholine, and that the challenge solution contained 18.8×10^{-4} µmoles as acetylcholine equivalents. Thus, the net release due to the action of streptolysin O was 9.0×10^{-4} µmoles. Correcting this value by the dry weight of the tissue (247 mg), and for the reaction period (90 sec), the unit rate of release was 2.5×10^{-3} µmoles acetylcholine/gram dry atrium/minute.

The Effects of Streptolysin O on the Isolated Ventricle.

Having examined, in detail, the characteristics of the response of the isolated atria to streptolysin O, it remained to demonstrate the effects of the toxin on the isolated ventricular myocardium.

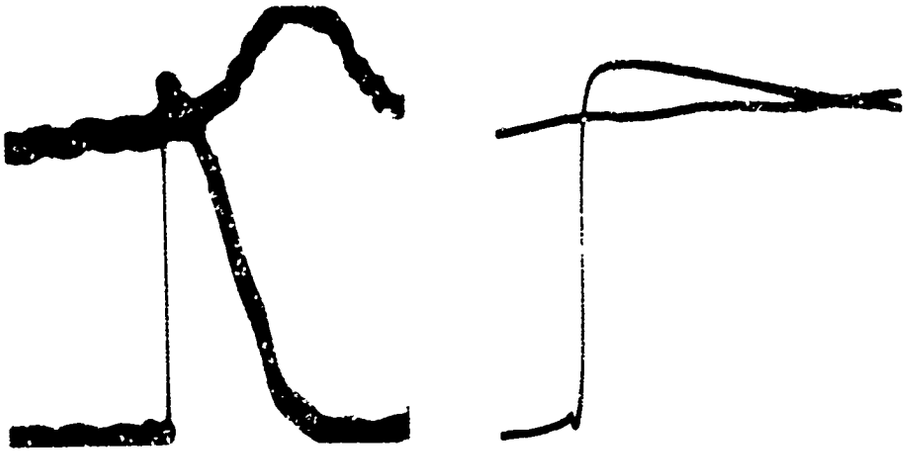
As mentioned above, electrically driven ventricle strips, obtained from streptolysin-intoxicated hearts in ventricular arrest, appeared to function normally. Similarly, the exposure of ventricle strips obtained from normal, untreated, hearts to high concentrations of active toxin (up to 400 HU/ml), established that streptolysin O had no detectable effect on the rate and amplitude of contraction or upon the configuration of the intracellular potential. The records of Figure 15 demonstrate that 200 HU/ml of reduced toxin produced no observable changes in the electrical or mechanical activity after 60 seconds (trace B); no changes occurred over a period of 30 minutes. In addition, as shown by trace C, this preparation was completely insensitive to acetylcholine; concentrations in excess of 1.4×10^{-5} *M* failed to have any effect on the recorded parameters.

The Significance of Acetylcholine Release to the Cardiotoxicity of Streptolysin O.

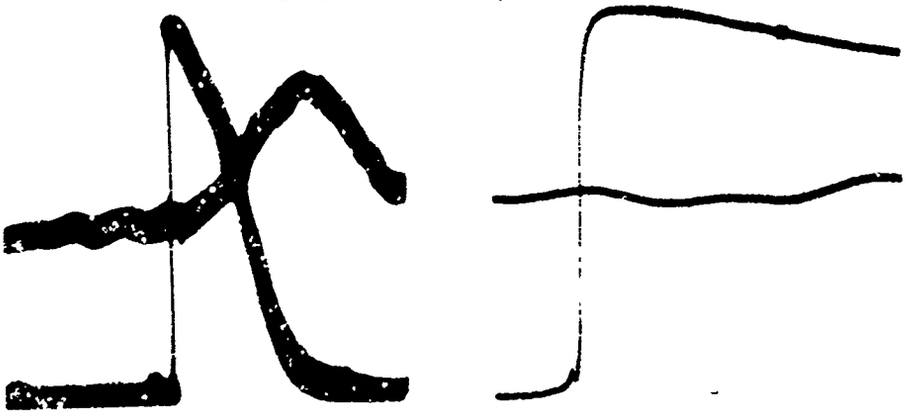
Having demonstrated that acetylcholine could be released from atria by streptolysin O and that the liberation of this transmitter substantially accounted for the functional changes observed in this tissue, it was appropriate to consider the contribution of this phenomenon to the toxicity of streptolysin O in the intact organ. For this purpose we made a detailed analysis of the time-courses of rate, amplitude, and perfusion flow changes in whole hearts subjected to physiologically "equivalent" doses of acetylcholine and streptolysin O in the presence and absence of atropine and eserine.

Figure 15. Oscillographic recordings of the contractions and intracellular potentials of an isolated ventricle strip preparation before challenge (A), 60 seconds after challenge with streptolysin O (B), and 60 seconds after challenge with acetylcholine (C). 37°C.

A. CONTROL



B. 60sec After SLO(200HU/ML)



C. 60sec After ACh(1.4×10^{-5}M)

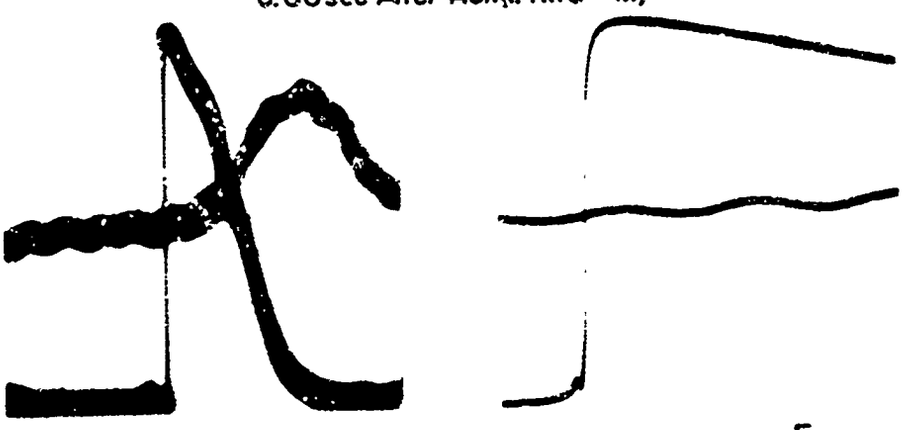


Figure 16 compares the effects of two concentrations of streptolysin O with their functionally equivalent doses of acetylcholine. There is a striking similarity between the transient depression of rate and amplitude resulting from acetylcholine administration and the initial phase of the toxin effect. The difference between the actions of the two agents lies in the recovery phase. The recovery of the acetylcholine-treated hearts was complete, whereas the toxin-treated organs recovered only partially, and then declined irreversibly.

The effects of atropine and eserine on the response of the whole heart to streptolysin O are demonstrated in Figure 17. The initial rate of decrease of amplitude produced by 132 units of active toxin was accelerated in the presence of 6.9×10^{-7} *M* eserine, whereas atropine (1.4×10^{-6} *M*) blocked this initial phase even when the streptolysin O dose was doubled. Although the pharmacological agents greatly influenced the course of the response in its initial stages, they had no effect on the rate of the irreversible decline of the terminal phase as evidenced by the convergence of the three curves of Figure 17 at about 4 minutes. In addition, these agents had no measurable influence over the changes in perfusion flow-rate produced by challenge with the toxin.

The Effects of Serotonin on Cardiac Tissues of the Guinea Pig.

Since certain anti-serotonin agents were protective against the lethal toxicity of streptolysin O in rabbits and mice(47), the possible

Figure 16. A comparison between the actions of streptolysin O and acetylcholine on the isolated heart at 37°C. Two concentrations of toxin (B and D) are compared with their functionally equivalent doses of acetylcholine (A and C).

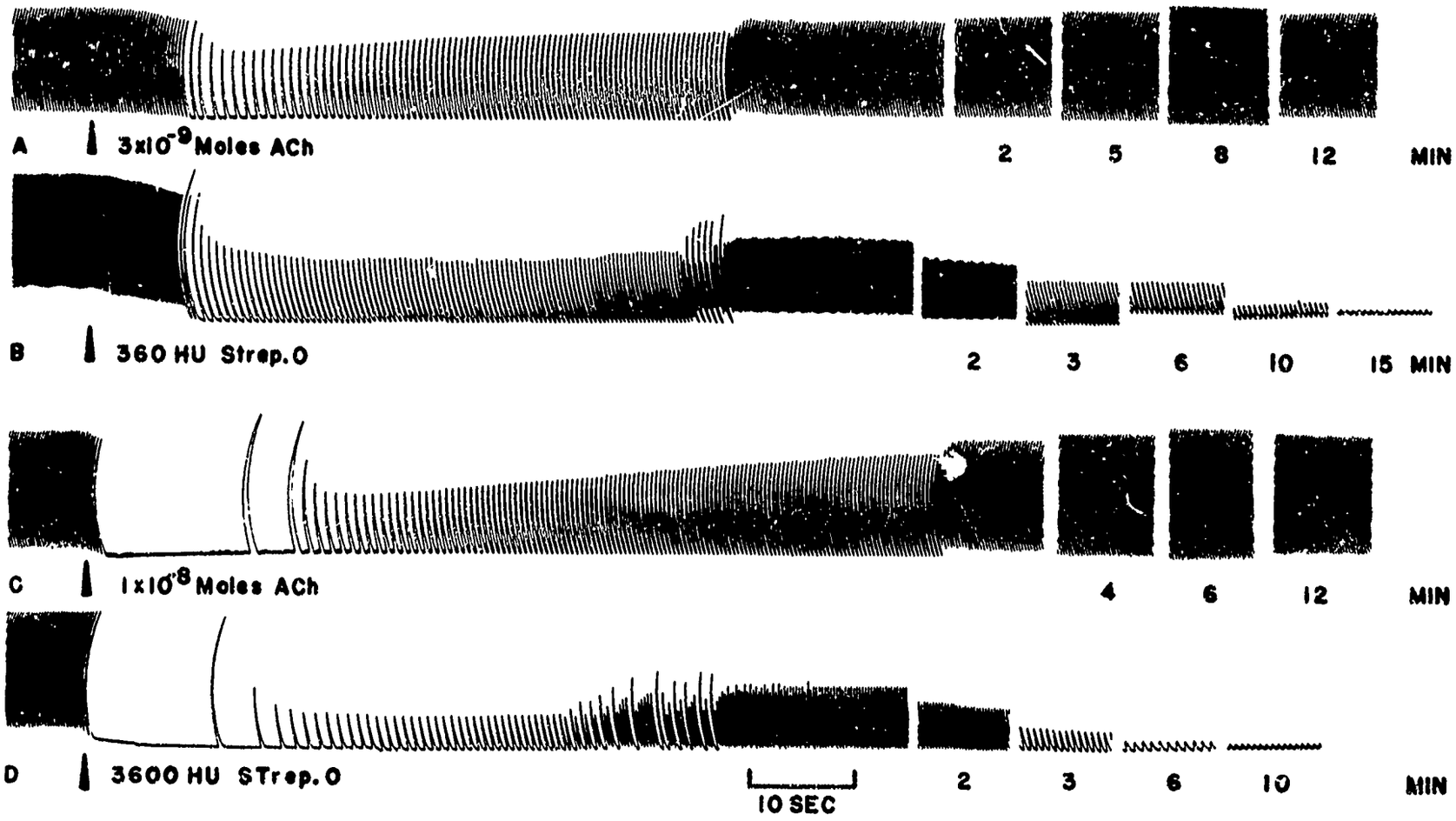
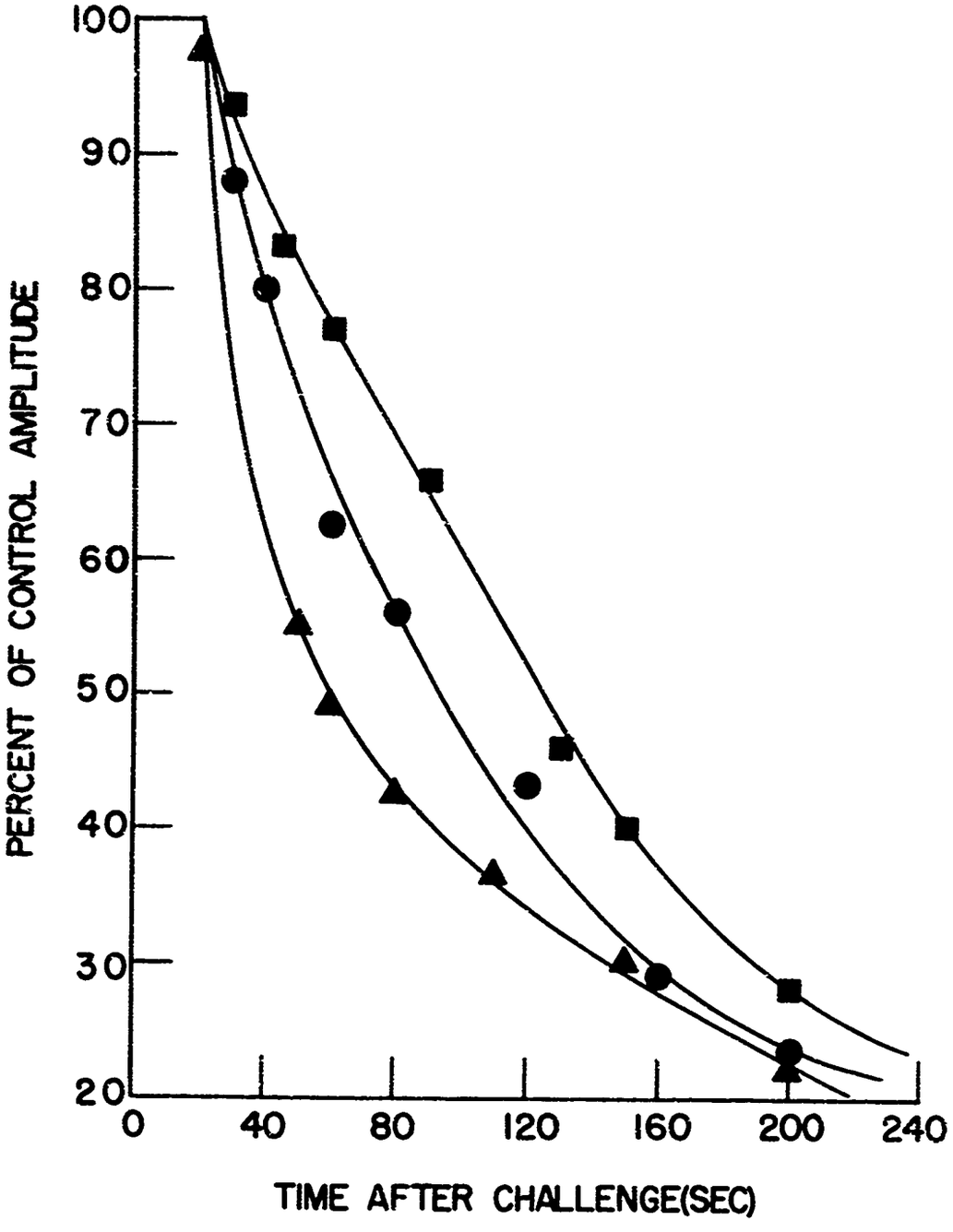

A
3x10⁻⁹ Moles ACh
2
5
8
12
MIN
B
360 HU Strep.O
2
3
6
10
15 MIN
C
1x10⁻⁸ Moles ACh
4
6
12
MIN
D
3600 HU STrep.O
10 SEC
2
3
6
10
MIN

Figure 17. The initial phase of the response of the isolated heart to 132 HU of streptolysin O (●——●). The effect of this same dose when administered in the presence of 6.9×10^{-7} *M* eserine (▲——▲), and double this dose when given in the presence of 1.4×10^{-6} *M* atropine (■——■). 37°C.


100
90
80
70
60
50
40
30
20
PERCENT OF CONTROL AMPLITUDE
0
40
80
120
160
200
240
TIME AFTER CHALLENGE(SEC)

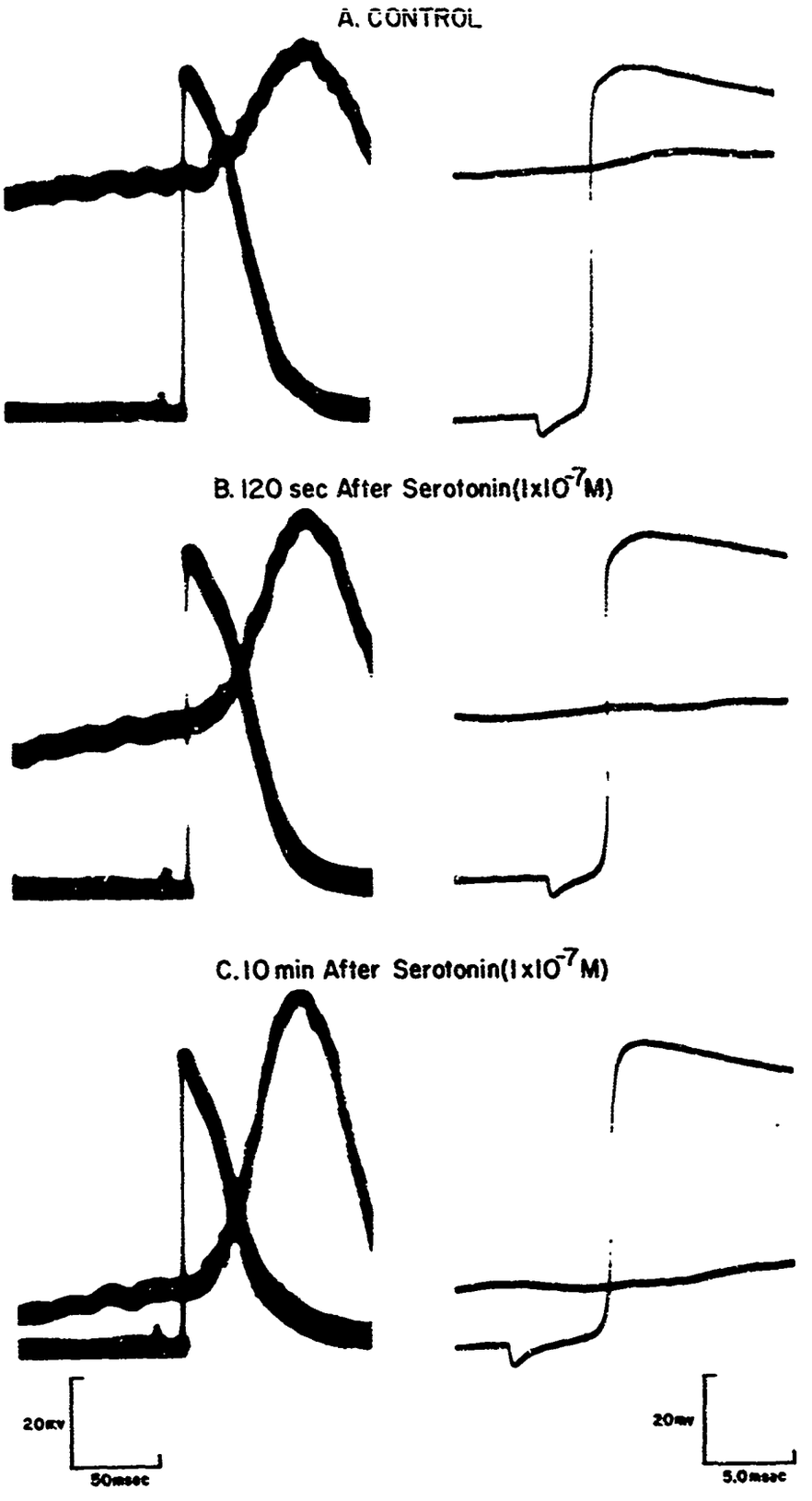
role of serotonin in the *in vitro* cardiotoxicity of streptolysin O for the guinea pig heart was investigated.

Very high concentrations of serotonin creatinine sulfate (2.0×10^{-4} *M*) applied to the isolated, perfused whole heart produced a slight, fully reversible, decrease in rate and amplitude of contraction, accompanied by a marked *increase* in the perfusion rate (up to 100%). The serotonin blocking agent, UML-491, modified this response but had no effect on any part of the response of the whole heart to 240 units of streptolysin O.

Electrically driven, isolated atria responded only to very high concentrations of either serotonin creatinine sulfate or *n*-acetyl serotonin. For example, a concentration of 4.7×10^{-6} *M* of the *n*-acetyl derivative produced a rapid, 110%, *increase* in amplitude which continued until renewal of the medium. Even such high concentrations failed to produce any measurable alterations in the intracellular potential. UML-491 (1.25×10^{-2} mg/ml) protected the preparation against these serotonin doses but did not alter its response to 240 HU/ml of streptolysin O.

Similarly, large doses of these compounds *increased* the amplitude of contraction of the electrically driven, isolated ventricle strip, but had no effect on the configuration of the action potential. Figure 18 shows the results of exposing such a preparation to 1×10^{-7} *M* serotonin for 120 seconds, and 10 minutes; the marked potentiation of the contraction is not accompanied by observable electrical changes.

Figure 18. Oscillographic recordings of the contraction and intracellular potential of the isolated ventricle strip before (A), 120 seconds after (B), and 10 minutes after (C), challenge with 1×10^{-7} *M* serotonin creatinine sulfate at 37° C.


A. CONTROL
B. 120 sec After Serotonin(1×10^{-7}M)
C. 10 min After Serotonin(1×10^{-7}M)
20mv
50msec
20mv
5.0msec

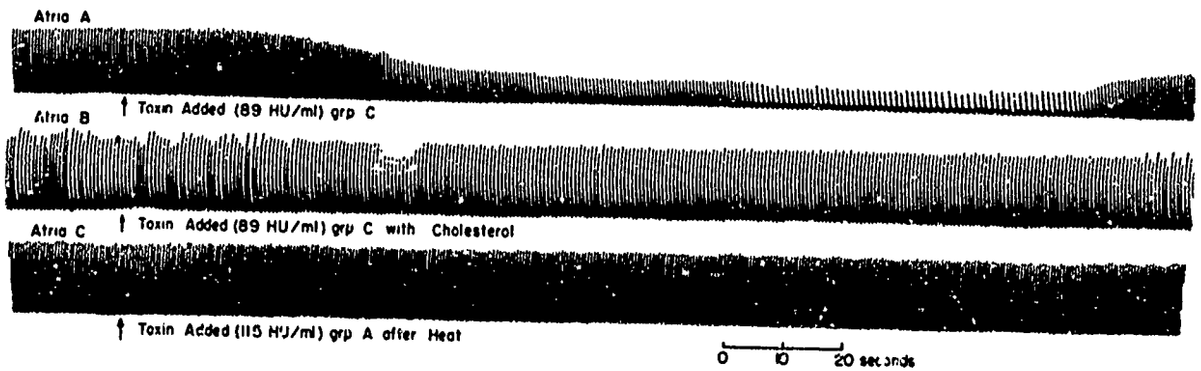
Evidence that the Cardiotoxic Factor is Streptolysin O.

Although the streptolysin O preparations used were highly purified, they were known to be contaminated with small amounts of one or two other antigens (46, 40). Certain properties of streptolysin O were used to ensure that the effects considered in this report were owed to the action of that molecule (36, 51, 46).

Inactivity of the Oxidized State. In tests on the whole heart and isolated atria, reversibly oxidized group A and group C toxins, having potential hemolytic potencies of up to 1200 HU/ml, produced none of the changes in rate and amplitude of contraction which characterized the actions of the same concentration of the reduced material. Similarly, bubbling oxygen through streptolysin O previously shown to be fully active, reduced the potency of the toxin to a degree dependent upon the extent of oxygenation.

Inhibition by Cholesterol. Aliquots of the activated toxin were incubated with the cholesterol suspension, then tested on the isolated atria. Figure 19 shows that such a treatment completely inhibited the effects of 89 HU/ml of the toxin preparation (Atria B) while the same dose of untreated toxin produced the usual mechanical changes (Atria A). Incubation of atria with cholesterol suspension, followed by thorough washing, failed to protect the tissues from subsequent challenge with active streptolysin O.

Figure 19. The ability of cholesterol and heat to inhibit the activity of streptolysin O tested on isolated atria. A dose of 89 HU/ml of active toxin, which elicited the response seen in the record of Atria A, produced no effect when first incubated with cholesterol (Atria B). Heating the toxin first (56°C, 10 min) also destroyed its depressant effects (Atria C). 30°C.


Atria A
Atria B
Toxin Added (89 HU/ml) grp C
Atria C
Toxin Added (89 HU/ml) grp C with Cholesterol
Toxin Added (115 HU/ml) grp A after Heat
0
10
20 seconds

Heat Inactivation. The thermolability of our toxin preparations was demonstrated by heating the toxin-buffer solutions (56°C for 10 minutes) prior to activation with cysteine. In the example of Figure 19 (Atria C) heat-treated toxin produced no response when added to the bath (final concentration 115 HU/ml) while untreated toxin of the same dose produced the expected effects.

Neutralization by Specific Antibody. In these studies, normal pooled human γ-globulin at a concentration of 160 mg/ml was used. An aliquot of 0.5 ml of group C toxin, activated with cysteine and diluted to give a final concentration of 5880 HU/ml, was incubated at 24°C for 5 minutes with 0.25 ml of the human γ-globulin. The data in Table III demonstrate that the severe depression of atrial contraction produced by three doses of untreated toxin were completely prevented by previous incubation of the toxin with antibody. Controls, in which streptolysin O was similarly incubated with human serum albumin or bovine γ-globulin before testing on atria, showed that these proteins provided no significant protection against the effects of the toxin.

DISCUSSION

These studies, performed with rather highly purified toxin preparations, confirm the *in vitro* findings of Kellner, *et al.* (51) who demonstrated the ability of microgram quantities of reduced streptolysin O to induce cardiac standstill in isolated mammalian hearts. In addition, however,

TABLE III

Neutralization by Antibody (IHG) of the Toxicity of Streptolysin O On the Isolated Atria

Expt. No.	Atria No.	Strep. O (HU/ml)	Protein[1] (mg/ml)	% Changes in Amplitude (ΔA), Rate (ΔR)
	1	112	-	- 64 ΔA - 17 ΔR
I	2	112	2.36 IHG	no change
	3	-	2.36 IHG	no change
	4	136	-	- 57 ΔA - 5 ΔR
II	5	136	1.34 IHG	no change
	6	136	0.43 BGG	- 48 ΔA - 0 ΔR
	7	184	-	- 88 ΔA - 77 ΔR
III	8	184	1.34 IHG	no change
	9	184	1.49 HSA	- 48 ΔA - 0 ΔR

[1] IHG = Immune Human Globulin, E. R. Squibb and Sons, New York

HSA = Human Serum Albumin, Cutter Laboratories, Berkeley, California

BGG = Bovine Gamma Globulin, Armour Pharmaceutical Co., Kankakee, Illinois.

it has been shown that the response of the whole heart to this toxin consists of two separate and distinct phases. There is initially a rapid, transient decrease in the rate and amplitude of contraction associated with the release of acetylcholine from the atria. These events are superimposed on the second phase, a gradual, irreversible decline in ventricular contraction.

In the whole heart, the initial phase of the response to streptolysin O appears to be entirely atrial in origin and exactly parallels the action of the toxin on the isolated atria. Both the whole heart and the atria show an immediate, dose-dependent, decrease in rate and amplitude of contraction, followed by spontaneous recovery. This is complete in the isolated atria, but in the whole organ, is interrupted by the gradual and irreversible ventricular decline. The transient depression can be accounted for by the toxin-induced release of acetylcholine since (i) there is excellent correspondence between the actions of streptolysin O and acetylcholine on the whole heart and on the isolated atria; (ii) the increase in the rate of repolarization of the atrial action potential caused by streptolysin O is characteristic of the action of acetylcholine; (iii) atropine modified the initial phase of the reaction of the whole heart to streptolysin O and completely protected the isolated atria from its effects; (iv) eserine aggravated the transient depression in the whole heart and isolated atria, and, finally, (v) acetylcholine was pharmacologically identified in the solutions bathing multiple atria challenged with streptolysin O.

The initial difficulties in recovering acetylcholine from atria challenged with streptolysin O can be understood after consideration of the kinetics of the release and destruction of acetylcholine. The release of endogenous transmitter occurs in close proximity to the cholinergic receptors so that relatively small amounts of released material produce rapid and profound changes. This action is necessarily transient in nature, however, because of the rapid enzymatic hydrolysis by the potent acetylcholinesterases. Any transmitter, which escapes destruction, encounters barriers to diffusion from its site of release and action to the bulk phase medium. Thus, only a very small percentage of the acetylcholine released by streptolysin O can be recovered from the solution surrounding the tissue. Conversely, these same factors limit the effectiveness of applied exogenous acetylcholine by sharply reducing the amount of intact, active material which reaches tissue receptors. For this reason, excessive amounts of the compound must be added to the bulk phase in order to reproduce the effects of locally released material.

These problems were overcome by using large quantities of tissue, high doses of toxin, and by blocking the enzymatic destruction of acetylcholine with eserine. We were thus able to detect, biologically, a net release of 3.8×10^{-3} µmoles of acetylcholine/gram dry tissue from atria challenged with toxin. Over the 90 seconds reaction period, this represents a net release of 2.5×10^{-3} µmoles acetylcholine/gram dry

atrium/minute. The rate of destruction of exogenous acetylcholine, estimated from the data of Table II, amounts to 3.4×10^{-4} μmoles acetylcholine/gram dry atrium/minute. Although these quantities and rates are gross approximations of the actual acetylcholine release and destruction at the cellular level, they provide evidence of the formidable barriers to quantitative recovery.

The observed atrial tachyphylaxis to repeated doses of streptolysin O could have come about because of an interference with the tissue receptors for acetylcholine, or because of a defect in the apparatus concerned with either the metabolism or release of the compound. Preliminary experiments on this question indicate that there is no alteration in the tissue receptor for acetylcholine. The top curve of Figure 7 shows that there is no loss of sensitivity to repeated, large doses of the transmitter. Furthermore, experiments in which alternate doses of acetylcholine and toxin were given repeatedly, showed no change in the response to acetylcholine, while the responsiveness to streptolysin O declined. However, as these latter experiments were made with a rather low concentration of toxin, they were not sufficiently critical, and we are not yet able to distinguish with certainty among possible defects in acetylcholine metabolism, release, or receptor mechanisms.

The terminal phase of streptolysin O toxicity in the whole heart is a gradual loss of force of contraction. This defect apparently does not result from damage to the myocardial contractile mechanism itself,

but to the system which distributes the excitatory impulses. That the contractile mechanism is functional was established by the ability of isolated strips of myocardium from arrested ventricles of toxin-treated hearts to contract normally when artificially stimulated. In addition, high concentrations of streptolysin O had no toxic effects on isolated ventricle strips from normal hearts. This is in conformance with the findings of Goullet *et al.* (72) who observed only a transient, *positive*, inotropic effect in the isolated ventricle strip of the rat. The observation that, at ventricular standstill, the atria beat normally indicates that the sino-atrial node is functional. These findings, suggest that ventricular standstill, subsequent to streptolysin O administration, results not from damage to the contractile tissue of the ventricles, but to the atrio-ventricular node or other components of the conduction system. This conclusion substantiates the *in vivo* findings of Halbert *et al.* (46) whose electrocardiographic data from rabbits was characterized by conduction defects and ventricular automatism.

Although there is strong evidence to implicate acetylcholine as the agent directly responsible for the transient depression of the whole heart, the evidence is equally convincing that it plays no part in the ventricular depression of the irreversible terminal phase. The studies on the isolated intact heart demonstrated (i) that atropine and eserine had no effects on the ultimate decline of ventricular force of contraction, and (ii) that hearts challenged with large doses of

acetylcholine recovered fully from cardiac arrest and failed to show any of the long term effects characteristic of the terminal phase produced by streptolysin O.

Additionally, isolated ventricle strips from normal hearts show no electrophysiological or mechanical changes subsequent to huge doses of streptolysin O or acetylcholine. This is in conformance with previous work which established the lack of parasympathetic innervation of the ventricles and their insensitivity to acetylcholine (73).

The analysis of the cardiac response to larger doses of toxin is complicated by the immediate and permanent cessation of coronary perfusion. Figure 2 (dashed line) shows the decrease in contraction amplitude resulting from artificially blocking coronary perfusion. This was also shown by Kellner, *et al.* (51) who stopped coronary flow and noted a rapid decrease in ventricular contractile force which, however, recovered completely after restoration of normal perfusion. Thus, at high levels of streptolysin O intoxication, it is not possible to distinguish between the deleterious effects of anoxia, and the decreased ventricular activity owing to the action of the toxin on impulse conduction. Such a distinction, however, is possible with lower amounts of toxin which invariably lead to an irreversible decrease in ventricular function, though they produce only transient lowering of perfusion (ref. 51 and present work, Figure 12). In these situations, it is clear that

streptolysin O has an effect on ventricular contraction which is separate and distinct from its influence on coronary perfusion.

Contrary to the experience with intact animals (47), our work failed to produce any evidence that serotonin plays an active role in the *in vitro* cardiotoxicity of streptolysin O. On the contrary, the changes produced by the extremely large doses of the amine, *i.e.* (i) positive inotropic and chronotropic effect on atria, (ii) positive inotropic action on the isolated, driven, ventricle strip, and (iii) increased coronary flow in the whole heart, are opposite to the changes produced by the toxin. Furthermore, anti-serotonin agents appeared to have no effect against the actions of streptolysin O in the isolated atria or whole heart.

It is interesting to consider the results of these studies and those of Halbert (46) in relation to certain of the clinical manifestations of streptococcal infections and their sequelae in man. The electrocardiograms of patients suffering from scarlet fever, streptococcal pharyngitis, and rheumatic fever often show abnormalities characteristic of damage to the atrio-ventricular node or ventricular conduction system. The most common change is prolongation of the P-R interval, but partial atrioventricular heart block, atrioventricular dissociation, inversion of the T-wave, and bundle branch block are also encountered (74, 75). These observations are of interest in light of Halbert's *in vivo* electrocardiographic findings of conduction defects following injection of streptolysin O into rabbits, and our own evidence that the *in vitro*

toxicity of streptolysin O for the guinea pig heart results from damage to the conduction system. Since the toxin is known to be present during the course of rheumatic fever (76), the above findings are compatible with the concept that this molecule may be responsible for the conduction defects represented by the diagnostic abnormalities of the electrocardiogram.

PART II. Cardiac Anaphylaxis in the Guinea Pig

INTRODUCTION

General Considerations

Immunological mechanisms, although usually considered as acting to increase the resistance of an animal to disease or to help protect him against the effects of deleterious foreign substances, also include a number of conditions in which the animal is actually more, not less, susceptible to such agents. These conditions, called hypersensitivity states, are characterized by heightened reactions to an antigen, resulting in harmful effects on the body. Thus, an animal which has been injected with a harmless protein (egg albumin, for example) may actually become, not resistant, but highly susceptible to it (hypersensitive), so that a later injection of a small dose may kill him very quickly. In this way, also, an animal which has been infected with a disease-producing agent such as the streptococcus, although apparently more resistant to a subsequent infection, may be definitely less resistant than a normal animal to toxic proteins, like streptolysin O, which are elaborated by the bacterium.

The conditions of heightened susceptibility are closely linked with the protective mechanisms because both states are produced in most, if not all, cases by processes which show the closest similarities. They exhibit the same high degree of specificity, can often be shown to depend on the presence of antibodies, and often result from infection with a specific organism or from the exposure to a specific foreign substance

(77). Thus, the union of antigen and antibody *in vivo* which can have, and often does have, beneficial effects, may have harmful effects owing to disturbances of cells either in the immediate area in which the union takes place or remote from that area, or due to cellular damage caused by toxic substances released as a sequel of antigen-antibody combination. These manifestations of the hypersensitivity reactions are really side issues to the central fact of this combination. The neutralization of the antigen by the antibody, resulting in protection from the direct actions of the antigen, may go on while, independently, a hypersensitivity reaction also occurs. This has been shown schematically by Raffel (78):

(a) Acquired resistance:

antigen + antibody → neutralization of infectivity or toxicity of antigen

(b) Hypersensitivity:

antigen + antibody ------→ [neutralization, if it occurs with the antigen in question, is a side issue]

↓

union

↓

"harmful" effects on body tissues

Hypersensitivity reactions can be divided into two types: immediate and delayed (early and late). Only the first type is germane to our discussion here. Immediate hypersensitivity can be produced by ordinary antigens introduced in the usual ways. It is associated with circulating antibodies, and the sensitivity can be passively transferred by injecting the serum of a hypersensitive individual into a normal recipient. The reactions can be evoked only in vascularized tissues, and depend largely upon changes which occur in the blood vessels, smooth muscle, and collagen.

Anaphylaxis is one of a group of phenomena which fall under the general heading of immediate hypersensitivity. It was first described in 1902 by Portier and Richet (79) who injected dogs with extracts of sea anemones and mussels in an attempt to immunize the animals against extractable poisons of these animals. They found, however, that two or three weeks after injection of a primary sublethal dose, a very small second dose would be fatal. It appeared that instead of becoming immunized, the dog developed a condition just the reverse, *i.e.*, a hypersensitivity to the agent. The development of a harmful sensitivity to a relatively innocuous substance led these workers to describe the state as the antithesis of prophylaxis; therefore, they applied the word "anaphylaxis". This term has since been employed to denote acute shock-like reactions which may occur subsequent to the injection of antigen into hypersensitive man or other animals (78).

In Vivo Anaphylaxis

Early Observations. Although Magendie (80) first noted the sudden death of animals repeatedly injected with egg white in 1839, attention was first definitely drawn to the phenomenon now known as anaphylaxis by Portier and Richet (79). In the following year, Arthus (81) published an account of his observations on the hypersensitivity of rabbits to successive injections of horse serum. Although the first few injections were tolerated without difficulty, the fourth injection produced a mild reaction at the site of injection. This local reaction, now known as the "Arthus phenomenon", was also observed when the first few injections were made intraperitoneally and the subsequent ones subcutaneously. However, when Arthus administered the first doses subcutaneously and then injected a dose of serum intravenously, he observed serious disturbances and even death in the rabbits. Repeating his experiments with milk instead of serum, Arthus was probably the first to note the specificity of the anaphylactic response, when he stated that the rabbits sensitized to serum were not sensitized to milk, and *vice versa*.

Perhaps the first clinical studies of anaphylaxis were published by von Pirquet and Schick (82) in 1903. In these studies, which came about from their observations of patients receiving protective doses of immune equine serum as an antitoxin to diphtheria, they noted that their patients often reacted rapidly to a second injection of the serum. Later, von Pirquet introduced the word "allergy" as an all-inclusive term

denoting a condition of "altered reactivity" of the body to antigenic substances (78).

Anaphylaxis in the guinea pig has supplied the greatest amount of information on the nature of these reactions and has afforded the basis for many fundamental concepts of hypersensitivity (83). In 1904, Theobald Smith (84) noted that guinea pigs prepared by injections of diphtheria toxin-antitoxin mixtures promptly displayed fatal anaphylactic shock when injected several weeks later with normal horse serum. Otto (85) found that sensitization was independent of the toxin or antitoxin content of the injected material but was induced by horse serum alone. He was probably the first to note the phenomenon of desensitization when he observed that sensitized guinea pigs given a number of very small injections of horse serum withstood subsequent doses which ordinarily produced fatal anaphylactic shock. Other authors (86, 87, 88) have confirmed these findings and noted that if guinea pigs survived anaphylactic challenge, they remained unresponsive to the antigens for some time after the first challenge.

The amount of antigen required for sensitization varies widely according to the species of animal and molecular properties of the antigen; but, generally, all that is necessary is an amount sufficient to elicit antibody formation (77). Rosenau and Anderson (87) in 1906 were successful in sensitizing guinea pigs with as little as 10^{-6} ml of horse serum. However, with this dose, the sensitization was slight and

the subsequent anaphylactic reaction was not fatal; larger sensitizing doses gave more consistently fatal results. Wells (89) got definite sensitization of small guinea pigs with 5×10^{-8} grams of crystallized egg albumin, and this amount apparently approaches the lower limit.

The majority of the antigens used for sensitization have been proteins or carbohydrates. However, Landsteiner and Jacobs (90) induced anaphylaxis by injections of arsphenamine, and Landsteiner and Chase (91) were able to sensitize animals anaphylactically by the cutaneous administration of simple compounds such as picryl chloride. It is probable that such simple molecules combine with host proteins, and that it is the protein-hapten complex that actually sensitizes (77). Similarly, it has been shown that the anaphylactic reaction can be elicited by simple compounds. Landsteiner and van der Scheer (92, 93), and later others (94, 95) have been able to produce anaphylactic shock by injecting azodyes.

There is usually a latent period of a week or more between the initial (sensitizing) injection and the time at which a second dose of the antigen produces anaphylactic shock. This was recognized in the early studies of Otto (85) and Rosenau and Frost (87). Coulson, *et al.* (96, 97), and Kabat, *et al.* (98, 99) reported that the intensity of the anaphylactic reaction in guinea pigs depended on the amount of antigen used to sensitize the animals as well as the amount used to challenge the animals. Coulson and his colleagues found that the dose of antigen

required to produce a standard intensity of reaction increased as the amount of antigen used to sensitize increased; however, Kabat's group obtained conflicting results. Because of the uncertainties involved in attempting to use the sensitizing dose of antigen as a measure of the subsequent degree of sensitization, this discrepancy was not resolved until *in vitro* methods for studying these reactions were employed.

This is a good example of the limitations inherent in the studies of gross, systemic, anaphylaxis. This technique has indeed been useful in determining the immunologic responsiveness of a subject, the comparative characteristics of reactions in whole animals, and the effectiveness of prophylactic measures. However, its usefulness in quantitative studies of sensitization, the stoichiometry of antigen-antibody reactions, and the release of pharmacologically active materials, or in investigations of the complex mechanisms underlying the gross manifestations of anaphylaxis, is severely limited.

These defects were partly overcome when it was observed that sensitivity could be transferred from a sensitized animal to a normal recipient. This technique permitted closer control of the amount of antibody involved in the production of anaphylactic sensitization. Kabat, *et al.* (98, 99, 100, 101, 102) used this technique to study the amounts and kinds of antibodies required to produce systemic anaphylaxis in the guinea pig, and Pruzansky, *et al.* (103, 104, 105) refined these methods for their studies of the kinetics and dose-relationships in the

anaphylactic reaction in passively sensitized guinea pigs. However, the quantitative nature of the scoring system used to rate the severity of systemic anaphylactic reactions tended to mask the quantitative aspects of this phenomenon and there was still little that could be said about the basic mechanisms responsible for the outward manifestations of anaphylaxis.

In Vitro Anaphylaxis

Early Observations. Most of our present knowledge of the mechanism of anaphylaxis has been obtained through studies of the anaphylactic reaction in isolated tissues. Such tissues can be prepared either from actively or passively sensitized animals, or they can be passively sensitized *in vitro*. This technique permits the experimenter not only to control the sensitization and challenge procedures, but also permits him to measure the intensity of intermediate responses and processes. Many tissues have been used successfully for this purpose: the isolated gut, uterus, aorta, lung, tracheal rings, heart tissues, as well as isolated leukocytes, platelets, and mast cells.

Smooth muscle from a sensitized animal contracts when exposed to antigen *in vitro*. This phenomenon, historically referred to as the Schultz-Dale reaction, has been studied most often in the small intestine (106) and uterus (107) of the guinea pig, but has also been demonstrated in the arterial smooth muscle of this species, first by Grove (108) in

1932, and in the bronchioles and pulmonary arterioles of the rabbit lung (109, 110).

The Schultz-Dale technique dates from 1910 when Schultz (106) found that if an intestinal strip from a sensitized guinea pig was excised and mounted in a bath of oxygenated Ringer's solution, addition of the specific antigen to the bath would induce a contraction of the tissue. Although Schultz concluded that the isolated tissue was in itself supersensitive to the protein to which the whole animal had been sensitized, the experimental evidence was not firm since the horse serum which was used as the antigen also caused normal and desensitized muscle to contract.

Dale (107), who apparently started his work at the same time Schultz did, observed that an isolated uterus, prepared from a guinea pig which had survived an antitoxin test, contracted strongly when a normally innocuous concentration of serum was added to the organ bath. By thorough perfusion of the sensitized uteri until they were free of blood, and by use of a nontoxic protein such as egg albumin for the antigen, Dale was able to demonstrate that the immediate reaction between antigen and antibody in the guinea pig tissue took place on the responsive tissue and not in the blood, and that the anaphylactic reaction was not simply an exaggeration of the toxic action of fresh sera. In this way, he was able to give a better demonstration of tissue sensitization than was Schultz, and placed Schultz's discovery on a firmer basis. Dale's studies became the foundation for much of the experimental work done in

the field of anaphylaxis of isolated tissues, and established the virgin guinea pig uterus as the tissue of choice of later workers in the field of *in vitro* anaphylaxis.

In time, however, observations were reported on the use of intestinal strips as the test tissue. Friedberger and Kumagai (111) in 1914, apparently unaware of Schultz's work, used intestinal strips from several species in tests for sensitivity, but obtained no clear-cut responses. However, in 1916, Massini (112, 113) obtained definite *in vitro* anaphylactic responses with intestinal strips from sensitized guinea pigs. A series of papers published between 1927 and 1930 by Kendall *et al.* (114, 115, 116, 117) re-established the use of intestinal strips for demonstrating tissue anaphylaxis.

The Mechanism of Sensitization

Fundamentally, sensitization appears to be a process, related to adsorption (118 - 125), by which one of the partners of an immunologically specific pair becomes attached to a critical receptor on a target cell. The weight of evidence is that the attachment is of a rather restricted kind (121, 126, 127) and is usually limited to certain classes of γ-globulin molecules (126 - 131) or operational fragments (132, 133). Practically, sensitization is recognized by a physiological signal, often the release of histamine, whose intensity is a quantal function (134, 100, 135, 121, 118, 122, 136, 119, 123, 124, 125) of the immunological input as well as of the magnitu _ of the immunological

stimulus (137, 125) eliciting the reaction.

The Anaphylactic Antibody. Evidence is accumulating that several species, including man, produce a specialized immunoglobulin capable of sensitizing host tissues for systemic, local, and *in vitro* anaphylactic reactions. This immunoglobulin, the anaphylactic antibody, can be defined as an antibody capable of combining with certain target cells, primarily tissue mast cells, so that subsequent contact with antigen leads to alteration of these cells accompanied by release of pharmacologic agents (138). The anaphylactic antibodies of a given species appear to "fit" receptors (139, 140) on target cells of the host and, in some instances, those of closely related species, but do not "fit" receptors on cells of more distantly related species (141, 142). In some instances, however, guinea pigs may be sensitized for anaphylactic reactions with antisera from heterologous species. This has been demonstrated for antisera from rabbit, dog, monkey, and man (143). Recent studies have shown that the γG antibodies of the respective species are responsible for this cross-species sensitization (144), and that these antibodies do not sensitize the tissues of the species of origin. Benacerraf has attributed this ability of certain heterologous γG antibodies to sensitize the guinea pig to the chance occurrence of a site or configuration on these gamma globulin molecules which "fits" guinea pig mast cell receptors (138).

It is generally agreed that anaphylactic antibodies are capable of sensitizing mast cells, although it is uncertain whether other

cells are capable of being sensitized and subsequently responding to antigen. Antigen-induced release of vasoactive substances from mast cells sensitized with anaphylactic antibody does not require hemolytic complement (145, 146). The process of releasing these substances appears to be a physiological function (146, 147, 148) of mast cells involving the action of one or more enzymes (149).

The Anaphylactic Antibody of the Guinea Pig. While studying certain anti-hapten antibodies produced by hyperimmune guinea pigs, Ovary and Benacerraf (150) noted that such antisera contained two populations of precipating antibody directed against the same antigenic determinant, but differed in their electrophoretic mobility. The rapidly migrating antibody component was identified as γ_1, the more slowly migrating component as γ_2. Both γ_1- and γ_2-globulin fractions and purified γ_1 and γ_2 anti-hapten antibodies, were tested for their ability to sensitize perfused, chopped, well-washed normal guinea pig lung tissue. Tissues sensitized to γ_1, but not tissues sensitized to γ_2, antibodies released histamine upon challenge with antigen. These results and others suggest that a single type of γ_1 antibody molecule is responsible for mediating anaphylactic reactions in the guinea pig.

Rabbit Heterologous Sensitizing Antibody. Rabbit antibodies, in small amounts, are capable of sensitizing the guinea pig for local cutaneous and systemic anaphylaxis (126, 143). Specifically purified rabbit 7S γ_2 anti-hapten antibodies sensitize guinea pig skin for passive

cutaneous anaphylaxis (144), and sensitize chopped guinea pig lung *in vitro* for antigen-induced histamine release (151), but fail to sensitize the rabbit, itself (144).

Adsorption and Sensitization. The relationship between the physical uptake of antibody and the degree of sensitization has been examined in various laboratories (135, 121, 124, 125). The physical uptake appears to be independent of the source of the antibody. When guinea pig mesentery, sensitized with rabbit 7S γ_2, or sensitizing antibodies from monkey, dog, or human, was brought into contact with antigen *in vitro* or *in vivo*, mast cell degranulation occurred (152). Antisera from other species failed to mediate local, systemic, or *in vitro* anaphylactic reaction in the guinea pig (126, 143). Humphrey and Mota (126), using I^{131}-labelled antibodies, could detect no gross difference between those antibodies which sensitized and those which did not, either with respect to uptake on guinea pig mesentery *in vitro*, or retention in tissue after uptake *in vivo*. Further, these authors showed by means of labelled antigen that antibody adsorbed to guinea pig mesentery could combine equally well with antigen, whether or not the antibody came from a species capable of sensitizing the guinea pig for anaphylactic reactions. These findings support the statement made earlier that sensitization appears to require more than simple adsorption of antibody to target cells and may involve the interaction of specific configurations on certain antibody molecules with limited or unrestricted sites on target cells.

This specific configuration on the antibody molecule, which determines its sensitizing properties, resides in a particular segment of the molecule. When rabbit anti-hapten antibodies are digested with papain according to the method of Porter, three fragments are obtained: Porter Fractions I and II, now called the F_{ab} or antigen-binding fragments; and Porter Fraction III, now called the F_c-fragment. Of the three, only the F_c-fragment has been shown to sensitize guinea pigs for reverse passive reactions, indicating that the tissue-fixing site is localized in this segment of the rabbit 7S γ_2 molecule (132).

The physical binding of antibody to guinea pig ileum is not influenced by temperature in the range 20 - 37°C (119). It is dependent only on the concentration of γ-globulin in the bulk phase. The uptake of radioiodinated antibody by strips of ileum (120) or chopped lung (121) at a constant temperature occurs very rapidly, the relation between the concentration present in the bath and that adsorbed to the tissue being described by a simple Langmuirian isotherm (120). The pharmacological response (histamine release) also increases with the concentration of antibody but in quite a different way (120), which emphasizes that the degree of sensitization is not simply determined by the antibody load physically adsorbed to the tissue.

The velocity of sensitization has been shown to depend on the antibody concentration (123, 118) and temperature (119) and the degree of sensitization at a constant time is usually chacterized by a positive temperature coefficient.

King and Francis (135) showed that a fast initial adsorption of radioiodinated anti-bovine serum albumin by guinea pig lung at 0°C was followed by a slow uptake lasting for about 48 hours. They resolved the bound antibody into two components by elution. While the adsorption of the weakly combined fraction was not significantly affected by temperature and had no effect on the subsequent binding of antigen, the adsorption of the strongly binding component was shown to be profoundly influenced by temperature and was responsible for the interaction with antigen, which occurred at a constant molar ratio of unity.

Vurek, *et al.* (153) in their studies on guinea pig cardiac tissues showed that for these tissues, also, the physical uptake of specific γ-globulin by the tissue was determined by the concentration of γ-globulin in the bulk phase, and that the quantities taken up from solution at 32°C and 37°C apparently were equal. However, even though the various cardiac tissues showed essentially no difference in the physical uptake of antibody, when challenged with specific antigen they released widely varying amounts of histamine. Moreover, the degree and velocity of sensitization were shown to be dependent on the concentration of antibody and on the temperature, as well as on the proportion of specific to non-specific γ-globulin molecules.

Feigen, *et al.* (154) have recently reviewed the subject of *in vitro* sensitization and its dependence on temperature and antibody concentration. The velocity of sensitization is influenced initially

by the time of contact between the reactants, the process becoming independent of incubation time and continuing to completion after the tissue has been removed from the antibody solution. When the incubation time is constant, the rate of sensitization can be separately influenced by the antibody concentration and the temperature. The velocity of reaction reaches a maximum and becomes independent of antibody concentration. The maximum velocity varies with temperature in a way dependent on the activation energy of the system. The induction or "lag" period which elapses between the passive transfer of antibodies and the time at which a certain degree of reaction first becomes evident, is also a variable of the antibody concentration and temperature.

It appears that the degree and velocity of sensitization can be sufficiently characterized by three precisely defined variables if a fourth, presently undeterminable, is kept constant. Given a set of constant preadsorption conditions, the velocity of the reaction, hence its outcome at a given time, is predictable from the antibody concentration, the ambient temperature, and the time of exposure. The overall degree of the reaction appears to be determined by the state of the tissue receptors as influenced by the environmental conditions prevailing during the preparation of the tissue for the experiment as well as by those ambient during the adsorption and the post-adsorption phases.

Considering the overall thermal change it might have been expected that the reaction should have proceeded at a rate faster than that actually observed. The comparative slowness might suggest that

binding takes place at specific regions of the cell, in a very definite molecular arrangement, and since the magnitude of the overt process is determined by the number of these activated cells, the rate would be limited by the probability of a mating in which a molecule -- or a number of molecules -- can become attached to the cell in a useful way.

It must be remembered that similar events may also take place on the physiologically inert structures and that the disjunction between physical adsorption and fixation is not necessarily in the character of the surface reaction as it is in the events which succeed attachment.

Feigen (154) has proposed a kinetic model which accounts reasonably well for the salient features of sensitization, particularly the variation of the lag period and velocity with concentration and temperature. Although it is a first approximation, it is able to explain the lag period as a result of the competitive kinetics of adsorption and desorption during the first stage of the hypothetical reaction sequence.

Assuming that the process of fixation is based on a mechanism involving a cell attachment of a highly restricted kind, and considering that there may be relatively few useful configurations for such an attachment, or only a few molecules in a population capable of reacting, it is evident that the velocity with which the right number of critical attachments can be made would depend on the antibody load on a cell, and

the number of critically reacting sites available. The kinetics and equilibrium of such a reaction have been described by the simple form of the Langmuir theory: if there are S sites per cell of which a fraction θ is occupied, the equilibrium reaction is

$$k_d S\theta = k_a S(1 - \theta)C$$

in which C is the antibody concentration, k_a and k_d are the rate constants for adsorption and desorption, respectively. The fraction of the occupied sites will depend on the equilibrium constant and the antibody concentration since

$$\frac{\theta}{1 - \theta} = \frac{k_a}{k_d} C = K_e C.$$

If the number of critically reacting sites, X, has to adsorb antibody in a particular way to form X^* so that X^* will liberate histamine quantally when antigen is added, we see that the rate of the reaction $X \overset{k_3}{\rightarrow} X^*$ can be made dependent upon the number of sites already occupied by antibody if the velocity constant is a constant embodying these characteristics.

Gathering these reactions into the form of a Michaelis-Menten sequence

$$Ab + X \underset{k_2}{\overset{k_1}{\rightleftarrows}} AbX \overset{k_3}{\rightarrow} AbX^*$$

in which $k_1 = k_a$ and $k_2 = k_d$ and Ab and X are the concentrations of antibody, and critically reacting sites, respectively, and AbX that of the reacted sites, the formation of critically reacted sites, AbX^*, will be limited by the amount of Ab adsorbed, and its rate will be determined by the antibody concentration; if the concentration of antibody is low, very little AbX would be formed and the rate of formation of AbX^* might be very slow. The lag period is probably the physiological manifestation of these competitive kinetics; therefore, if the equilibrium constant varies in the usual way with temperature, or if k_3 is temperature-dependent, the lag period, even at low antibody concentrations, will be greatly shortened, and the amount of sensitization acheived in a given time significantly enhanced by increasing the temperature.

Mechanism of Anaphylaxis in the Guinea Pig.

It is now known that the combination of antigen and antibody on, in, or near certain cells starts a chain of reactions which culminates in the release of pharmacologically active materials, and it is the action of these materials on the smooth muscle and other tissues which produces the physiological symptoms of anaphylactic shock (155). The general picture of the mechanism of anaphylaxis that is emerging (149, 156) has been summarized by Boyd (77):

> A number of pharmacologically active substances may be released by the reaction. The relative roles of these vasoactive materials vary from one species to

another, depending on (i) the relative sensitivity of the various animals to the different agents, (ii) the abundance of the substances or their precursors in the tissues, (iii) the location of the sensitive tissues relative to the sites of antibody-antigen reaction, and (iv) the types of antibodies involved.

In the guinea pig, *in vivo* anaphylactic shock can exhibit different courses depending on the route of the challenging antigen. If the antigen is administered intravenously, dyspnea due to acute bronchiolar constriction is the predominant sign; at post-mortem examination the lungs are markedly distended and bloodless (157). That the lung is the "shock organ" and the circulatory collapse secondary, is shown by the fact that these same symptoms can be produced *in vitro* in the perfused guinea pig lung (107). If a large amount of the same antigen is administered subcutaneously or intraperitoneally, there results a protracted shock which leads to death over several hours (158, 159). Post-mortem examination implicates the abdominal viscera rather than the lungs.

The Role of Histamine. In 1932, Bartosch *et al.* (160) demonstrated the antigen-induced release of histamine from sensitized, perfused guinea pig lung, and in 1939, Code (161) detected the release of histamine *in vivo* during anaphylactic shock in the guinea pig. The initial conviction of many workers that histamine accounted for virtually all the manifestations of the anaphylactic reaction in the guinea pig has been supported by the finding that antihistaminics gave

significant protection (162, 163, 164), and by the demonstration that *in vivo* mast cell degranulation occurs during this reaction (165, 166).

The first indication that substances other than histamine might be involved came from the observation of Kellaway and Trethewie in 1940 (167) that the effluent from perfused, shocked lung contained a slow-reacting substance, which Brocklehurst (168, 169) subsequently demonstrated was resistant to inhibition by antihistamines. The importance of this substance (Slow-Reacting Substance-Anaphylaxis, or SRS-A) in guinea pig anaphylaxis is difficult to estimate; the isolated guinea pig bronchiole is rather resistant to its action (170), but intravenous infusion of a crude preparation does cause increased resistance of the lungs to inflation (171).

Campbell and Nicoll (172) reported in 1940 that anaphylaxis in nonperfused, guinea pig lung produced a substance which, unlike histamine or SRS-A, contracted the rat uterus. Recently, Brocklehurst and Lahiri (173) have shown, in the guinea pig, that bradykinin is produced *in vivo* during anaphylactic shock, especially in the protracted form; that a kinin-producing enzyme can be released *in vitro*, by antigen, from perfused shocked guinea pig lung; and that in protracted shock, the plasma substrate from which bradykinin is formed is depleted (174).

Although Humphrey and Jaques (175) have demonstrated the *in vitro* release of serotonin from guinea pig platelets by antigen-

antibody interaction, they found no evidence that the absence of platelets altered the picture of acute systemic anaphylaxis in this species. The guinea pig bronchiole is moderately sensitive to this amine (176), but the concentration of serotonin in guinea pig lung is negligible (177), and serotonin has not been detected in the effluent from perfused shocked guinea pig lung (170).

In summary, present evidence suggests that the acute anaphylactic reaction in the guinea pig which follows intravenous antigen is mediated by the action of histamine and possibly SRS-A on the bronchiole. Bradykinin may be implicated in the protracted shock syndrome which follows intraperitoneal antigen (149).

Some Factors Affecting Histamine Release.

Antigen Concentration. By studying the processes of sensitization and histamine release, *in vitro,* the relationship between the degree of sensitization and the dose of antigen used for challenge has been clarified, thus resolving the conflicting results of Coulson (96, 97) and Kabat (98, 99) mentioned earlier. Investigators using isolated systems have found that if a larger dose of antibody is used to sensitize the tissue, a smaller dose of antigen is subsequently required to produce a reaction of a given magnitude (178, 179, 180, 135, 181).

Working with tissues sensitized to a standard degree, it has been found that the magnitude of the anaphylactic response increases

monotonically with increasing concentration of the challenging dose of antigen until a maximum is reached; thereafter, the response may decline in severity with increasing antigen concentration. The increasing response with increasing antigen has been demonstrated in the case of the gut (137, 178, 120), in studies on guinea pig mast cells (165, 152), and in the passive cutaneous anaphylaxis reaction (182, 183). In the case where the antigen dose is very small, it is possible to produce a subsequent reaction by additional challenges with antigen (182, 179, 184, 185, 155). Liacopoulos *et al.* (137) found that high antigen concentration inhibited the Schultz-Dale reaction; the amount of antigen required to inhibit the reaction increased as the amount of antibody used to sensitize the tissue increased. By sensitizing the tissues to two antigens simultaneously, they showed that the inhibition was not simply due to a lack of reactivity of the tissue. Inhibition by high antigen doses was also found in the case of the guinea pig lung by Brocklehurst *et al.* (121). Liacopoulos *et al.* (137) have suggested that both low concentrations and very high concentrations of antigen react with tissue-fixed antibody to produce complexes which are unsuitable for the production of anaphylaxis.

Effect of Temperature. The observation that the anaphylactic release of histamine from guinea pig tissue (aorta) is influenced by temperature was first made by Schild in 1939 (186). A detailed study with lung tissue was carried out some years later by Mongar and Schild (187). Preheating lung tissue at temperatures greater

than 42°C for 25 minutes prior to reacting the tissue with antigen at 37°C, greatly reduced the histamine release. The rate of heat inactivation increased as the preheating temperature was elevated to 45°C. Inactivation was permanent at 45°C, whereas preheating at 43°C, was followed by some recovery on prolonged standing at 37°C. Mongar and Schild showed that the inactivation was due to an effect on the tissue and not on the antibody.

When antigen was added to sensitized tissue at 17°C there was no histamine release, but on warming the reaction mixture to 37°C, the histamine release varied with the duration of contact between tissue and antigen at 17°C. A contact period of 15 minutes at 17°C did not diminish histamine release on warming, whereas a contact period of one hour reduced the release by more than 80%.

These experiments demonstrated that: (i) the anaphylactic release of histamine from guinea pig lung requires activation of a factor that is heat labile in the precursor state, and (ii) contact of sensitized tissue and antigen at 17°C activates a labile, temperature-dependent factor which, though unable to act at 17°C, is gradually dissipated.

During these studies, Mongar and Schild (1957) observed maximal histamine release from sensitized lung tissue challenged at 40°C. From their data of histamine release in the range of 20°C to 40°C, they calculated a Q_{10} of 12 for this process.

Time Course of Histamine Release. The anaphylactic response of sensitized guinea pig tissues is characterized by a rapid liberation of histamine. Chakravarty and Uvnäs (188), Austen and Brocklehurst (189), and Austen and Humphrey (190), have established this fact for guinea pig lung. The release from chopped, sensitized lung tissue (189, 190) began within 10 to 15 seconds after addition of antigen, and two-thirds of the total amount to be released was in the supernatant by the end of the first minute. Similar release velocities have been reported by Mongar and Schild (191) for guinea pig uterus and aorta. Nielsen and Feigen (184) showed that the release of histamine from shocked ileal strips reached a maximum value in about 5 minutes after the challenge.

Other factors affecting histamine release have been studied on various guinea pig tissues and those of other species, but discussion of these factors is not relevant to the present work. A thorough review of these factors along with recent investigations into the mechanisms underlying anaphylactic histamine release has been written by Austen and Humphrey (149).

Anaphylaxis in the Isolated Heart.

It has been the general opinion that the circulatory irregularities associated with fatal anaphylactic shock are secondary manifestations, usually resulting from the asphyxia caused by bronchospasm. While this view, first proposed by Auer and Lewis (157) in 1910, is plausible in the case of the guinea pig, it cannot be held with equal strength in the case of the rabbit (192), or the dog (193, 194), neither of which dies

of bronchoconstriction.

The first direct proof of the involvement of the heart itself as a primary target organ in anaphylaxis is due to Cesaris-Demel (195) who, in 1910, showed that isolated hearts of immunized rabbits and guinea pigs would react specifically to antigens used previously to sensitize the intact animal. Cesaris-Demel recognized the nonspecific effects of the crude antigens he employed and therefore challenged his preparations with doses below those producing nonspecific reactions and rechallenged the tissue with the same dose to show that the organ had indeed been desensitized. In contrast to these early but excellently designed studies, most of those made between 1911 and 1938 are difficult to analyze immunologically because of the use of impure - and sometimes bizarre - antigens which could have produced nonspecific reactions (196). In addition, because of the ease with which tissue anaphylaxis could be demonstrated in the guinea pig gut (106) and uterus (107), the technically more difficult procedure of cardiac anaphylaxis has been neglected.

Studies on the course of anaphylaxis in the isolated heart, made in guinea pigs (195, 197, 198, 199), rabbits (195, 198, 199, 200, 201, 202), cats (199, 203), and rats (204), show that the severe disorganization of the heart beat, succeeding an effective dose of antigen, varies qualitatively with the degree of immunization of the host. According to the comparative studies of Andrus and Wilcox (199), the pattern of the cardiac reaction to an effective dose of antigen, as well as to histamine, in the case of the guinea pig and rabbit, is characterized by an increase

in the rate and amplitude of contraction, a delay in atrioventricular conduction, abnormalities of origin and spread of excitation in the ventricles, and an acute reduction in the coronary flow. The parallelism between the effects of histamine and anaphylactic shock (202) has been shown in many animals including the rat (204).

Feigen *et al.* (70) later confirmed the results of Wilcox and Andrus (193). Using the isolated, perfused heart of the guinea pig sensitized to ovalbumin, they noted that antigenic challenge was followed by an acceleration in heart rate, an increase in the amplitude of contraction, a decrease in coronary flow, and, in intense shock, an A-V conduction block. These same authors (70), by means of microelectrode techniques, also examined the effect of *in vitro* anaphylaxis on the electrical activity of isolated guinea pig atrial tissue. Although the atrial tissue was stimulated electrically at a rate exceeding the natural frequency of the pacemaker, antigen administration initiated a new spontaneous rhythm which at times progressed to fibrillation.

The perfusate obtained during the height of the anaphylactic reaction in the isolated guinea pig heart was shown to contain histamine by bioassay and chromatographic study (70, 122). That histamine could be released from guinea pig heart tissue by anaphylactic shock *in vitro* had been demonstrated previously by Schild (186), whereas both Brocklehurst (169), and Chakravarty (205), have observed SRS-A release. There is no evidence that serotonin or acetylcholine are released during *in vitro* anaphylaxis of the guinea pig heart. Since the changes in rhythm,

amplitude, or coronary flow characteristic of anaphylaxis in the perfused guinea pig heart or the isolated atrial tissue can be produced by histamine alone, it has been proposed that this material is the primary mediator (70, 198).

MATERIALS AND METHODS

Immunologic Reagents

Antigen. The antigen used for the immunization of rabbits and for the challenge of sensitized guinea pig tissues was six-times crystallized ovalbumin prepared according to the method of Kekwick and Cannan (206).

Antibody. Rabbit antibody was prepared against the ovalbumin obtained as described above. The rabbits were given intravenous injections of the antigen twice weekly for six weeks. On the ninth day following the last injection, the rabbits were bled by cardiac puncture and the blood allowed to clot for several hours at room temperature followed by 12 hours in the cold. The antiserum was then separated from the clot, and the γ-globulin fraction precipitated by a three-fold treatment with one-third saturated ammonium sulfate. The precipitate was redissolved in 1% saline and dialyzed against saline until the dialysate was sulfate-free. Such γ-globulin preparations were stored in 10 ml serum bottles at 20°C until use.

The precipitating titer of these antibody preparations was measured using the micro-precipitin method of Lanni (207). For the work reported in these studies, the γ-globulin fraction employed had a specific antibody content of 32%.

Other Reagents.

Nutrient medium. All of the work reported in this section was performed on tissues surviving in Chenoweth's solution (68) containing 2 ug/ml semicarbazide as a histaminase inhibitor.

Histamine Standards. Histamine standards were prepared from histamine diphosphate (Nutritional Biochemicals, Cleveland, Ohio); a stock solution of 1×10^{-3} *M* was prepared in 1% saline and the standards made by dilution.

Experimental Animals.

Normal male guinea pigs, ranging in weight from 300 to 700 grams, were used in these studies. Actively sensitized guinea pigs were prepared by two intraperitoneal injections of 20 mg of ovalbumin, given on successive days; these animals were used within 18 to 50 days after the second injection. Guinea pigs were passively sensitized *in vivo* by an intraperitoneal injection of 2 mg of specifically precipitable antiovalbumin (γ-globulin) given 12 hours prior to the experiment.

Preparation of Tissue.

The experiments forming the basis of this section were made on

whole hearts, isolated atria and, in certain cases, on minced tissue. Regardless of the subsequent experimental maneuvers, all tissues were prepared according to the general procedure described in the "Materials and Methods" of Part I.

For the determination of the wet-to-dry weight ratios of atria, aortae, and ventricles, these tissues were surgically separated from whole hearts prepared and equilibrated in the standard manner. The anaphylactic histamine release of these three cardiac components was studied on tissues obtained in the same way and then minced into vessels containing Chenoweth's solution.

The bulk of the work reported in this section involved the factors affecting the anaphylactic histamine release from atrial tissue in the form of whole atrial pairs, individual left and right atria, and fragments of left and right atria. Intact atrial pairs were obtained from whole hearts of normal or sensitized animals, in the prescribed manner. Left and right atria were surgically separated by dissecting the pair above the ventricular septum, and atrial fragments were prepared from these. In all of these studies on atrial tissues, the individual atria, or portions thereof, were fitted with small loops of nylon thread at either end. One loop was attached to the hook on the end of a glass rod, the other loop was used to secure the tissue to the rod. In this way, small pieces of tissue could be readily maneuvered, and matched fragments from the same heart could be kept track of.

In Vitro Sensitization. The effect of antigen concentration on the release of histamine from isolated atria was studied on tissues sensitized *in vitro* by incubation in a 100 ug/ml solution of antiovalbumin for 60 minutes at 37°C.

Challenge. Whole hearts were challenged by the administration of a standard concentration of antigen into the main standpipe. Isolated tissues were challenged by incubation for a standard time and temperature in measured aliquots of antigen solution made up in oxygenated Chenoweth's solution containing 2 ug/ml semicarbazide. In the studies of the effect of temperature on atrial histamine release, seven temperatures were tested simultaneously. A thermostatically-controlled water bath was set at one of the seven temperatures; incubation and challenge vessels containing antigen-free Chenoweth's or a solution of antigen in Chenoweth's, respectively, were fitted with polyethylene bubblers and placed in the bath. The tissues, mounted on the glass rods, were first placed in the incubation solution, and then, at the preselected time, transferred to the challenge solution.

The technique of superfusion was used in experiments designed to follow the time-course of histamine release and to correlate this output with functional events in the atria. By this method, it was possible to control the perfusion rate of antigen and to collect small samples of perfusate, while recording the changes in amplitude and rate of atrial contraction which occurred as a consequence of challenge. The superfusing fluid was first passed through a sample warmer and then dripped over the

atria at a known rate. Atrial contractions were recorded by means of a Statham G-7A transducer, Statham G-18 control box, and a Dynograph pen-writing oscillograph. Such preparations were quite stable and retained 70% of their amplitude after 10 hours at 37°C.

Estimation of Histamine.

The chemical estimation of histamine as made in our laboratory combines the procedure of McIntire *et al.* (208) for its separation, with that of Shore *et al.* (209) for its fluorometric estimation.

The separation procedure (208) makes use of the fact that histamine can be driven into n-butanol from an alkaline aqueous solution, and reversibly and specifically adsorbed onto a specially treated column material. Accordingly, aliquots of the aqueous supernatants or perfusates from the anaphylactic reaction systems were shaken with n-butanol and the butanolic phase passed through columns packed with cotton-acid-succinate (210, 123). The adsorbed histamine was liberated with 0.2 N HCl and returned to the alkaline state with 2 N NaOH.

The alkaline column eluate was assayed for histamine by the method of Shore *et al.* (209) which depends on the reaction of histamine with o-phthalaldehyde (OPT). The eluates were allowed to react with the OPT for 4 minutes, and the reaction stopped by addition of 3 N HCl. The fluorescence intensity of the OPT-histamine fluorophore was measured on a Turner Model 111 Fluorometer (G. K. Turner Associates, Palo Alto, California), using an excitation wavelength of 360 mμ, and an emission

wavelength of 450 mμ. Standardization functions were linear between 5×10^{-8} and 5×10^{-6} *M* histamine. Histamine determinations are expressed either as moles per gram of fresh tissue, or as moles per gram of tissue dried to constant weight at 105°C.

RESULTS

The Distribution of Histamine in the Cardiac Tissues of the Guinea Pig.

Studies were made to determine the weight-fractions of the major components of the guinea pig heart, and the contribution by these separate tissues to the overall anaphylactic histamine release of the whole heart.

Composition of a "Standard" Heart. Whole hearts, prepared in the usual way and perfused with Chenoweth's solution until free of blood, were dissected into three components: the aortic stump, the atria, and the ventricles. These tissues were blotted gently on filter paper, placed in tared weighing bottles and their wet weights measured immediately. The bottles were then placed in an oven at 105°C and the tissue dried to a constant weight. The ratios of wet-to-dry weights are given in Table IV. The dry weights were also used to determine the relative composition of a "standard" dry heart by calculating the weight-fraction of each of the three components (Table IV).

Distribution of Anaphylactically Released Histamine. Measurements of anaphylactically releasable histamine were made on the separated tissues from hearts of 12 actively immunized guinea pigs. The hearts

TABLE IV

Contribution of Anaphylactically Released Histamine by the Separate Cardiac Tissues to the Overall Release by the Whole Heart

Tissue	fresh tissue weight / dry tissue weight	n*	% of dry weight of "standard" heart	Anaphylactic Histamine Release by Actively Sensitized Tissues: moles/gram dry tissue ($\times 10^{-7}$)		
				Per gram of Tissue	n*	Per gram of Whole Heart
Atria	5.47 ± 0.31**	25	11.8	4.50 ± 0.22**	12	0.53
Aorta	3.36 ± 0.53	22	6.2	1.20 ± 0.26	12	0.07
Ventricles	6.10 ± 1.50	18	82.0	0.49 ± 0.04	12	0.40
Whole Heart	5.90 ± 2.00	18	100.0	-	-	-

*n = number of tissues

**Standard error calculated by the range method

were first perfused and the atria, aortae, and ventricles then dissected, and separately minced into vessels containing oxygenated Chenoweth's solution. The tissues were challenged by incubation for 15 minutes at 37°C in a 1.0 mg/ml solution of ovalbumin. The solutions were filtered, an aliquot saved for histamine analysis, and the tissue fragments dried to constant weight. The mean values for anaphylactically releasable histamine, given in Table IV, show that the atria, which comprised only 12% of the dry weight of the whole heart, accounted for about 53% of the histamine released during anaphylaxis of the intact organ.

The Distribution of Histamine in the Guinea Pig Atria.

Since a number of the projected investigations involving the anaphylactic release of histamine required that control tissues be run simultaneously with the test tissues, the atria appeared to be ideal since one atrium could be used as a control for the other. For this reason, a comparative study was made to determine the properties of the two atria with respect to their anaphylactic release of histamine.

The Anaphylactically Releasable Histamine of the Left and Right Atria. The atria from 25 actively sensitized guinea pigs were prepared in the usual way, surgically separated, and the two halves treated identically. After a 30 minute incubation in oxygenated Chenoweth's solution at 37°C, the atria were challenged at the same temperature for 10 minutes in a 0.1 mg/ml solution of ovalbumin. The tissues were saved for dry weight determinations, and the supernatants chemically assayed for histamine. Table V, which presents the results of these

TABLE V

Comparison of Anaphylactic Histamine Release by Left and Right Atria*

Number of Hearts	Mean Anaphylactically Released Histamine** Moles/gram dry tissue ($\times 10^{-7}$) Left	Right	Ratio Left/Right
6	2.97	5.78	0.52
6	3.17	7.01	0.47
6	1.86	4.14	0.45
4	3.43	5.93	0.58
3	2.35	4.70	0.50
Mean Ratio:	0.50***		
Std. Error:	+ 0.03		

* All tissue actively sensitized

** "Anaphylactically Released Histamine" represents the histamine released by a 10 minute challenge with 0.1 mg/ml ovalbumin at 37°C.

*** All statistics performed on $\log_{10}$ data; antilog data given

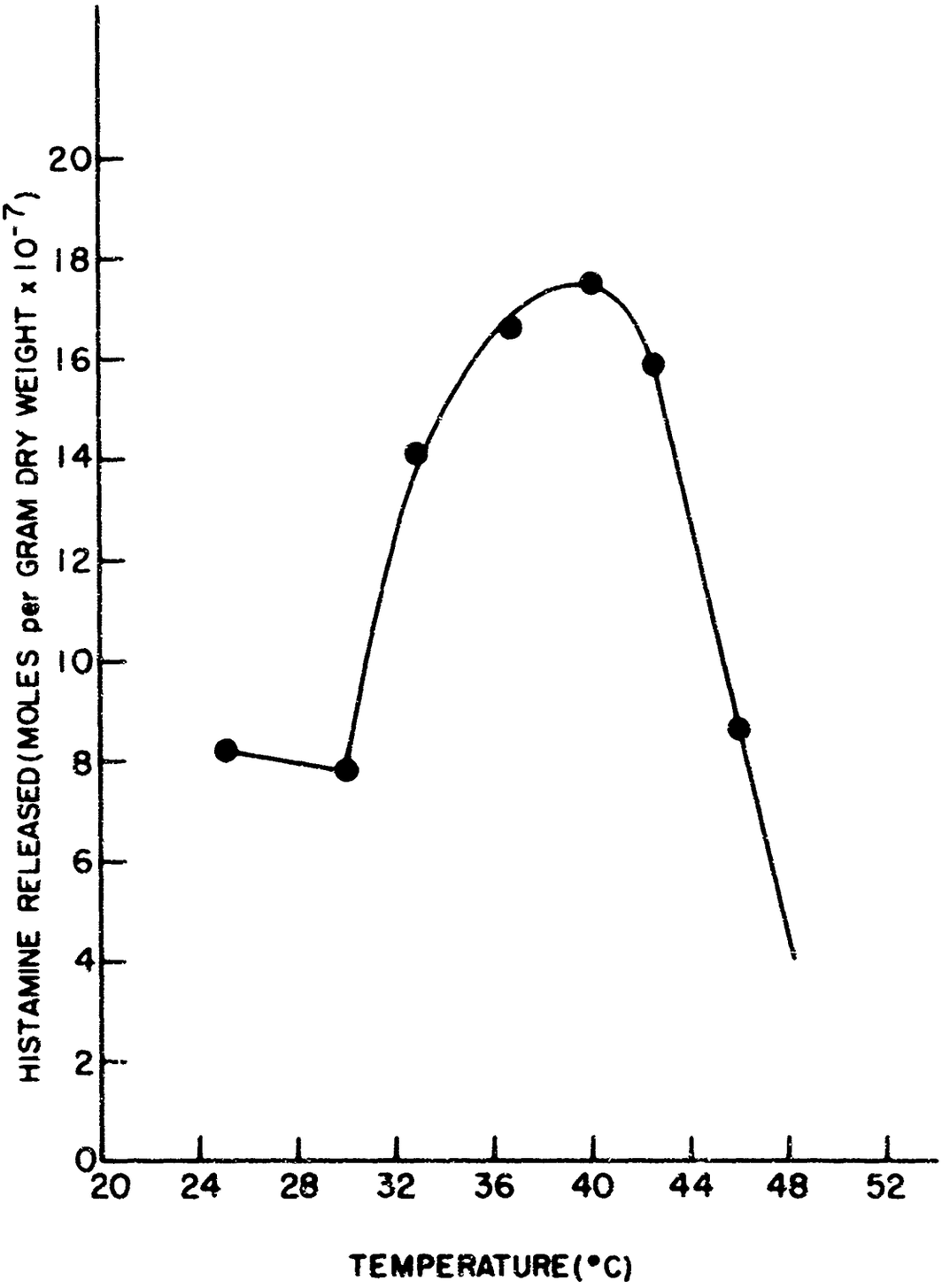
studies, grouped by experiment, shows that the right atrium, in response to the specific sensitizing antigen, released twice as much histamine as did the left atrium challenged under identical conditions.

The following experiments were designed to determine if this large discrepancy in the anaphylactic histamine release between the two atria could be accounted for simply by a difference in their stores of readily releasable material.

The Total Releasable Histamine of the Left and Right Atria. For these purposes, the "total releasable histamine" was defined as the sum of the histamine released by specific antigen and that subsequently released by acid extraction. Because of the unphysiological nature of the acid treatment, it was first necessary to investigate the temperature dependence of this extraction procedure.

Atria, obtained from seven normal guinea pigs, were incubated for 30 minutes in 0.01 N HCl at one of seven temperatures between 25 and 46°C, and the incubation solutions analyzed for histamine. Figure 20, which presents the results of these extractions, shows that this procedure is highly temperature-dependent with optimum histamine release occurring at 40°C. In further tests, a total of six atria extracted at 37°C averaged 16.6 x 10^{-7} moles of histamine released per gram dry tissue. The succeeding acid extractions were done at this temperature.

Figure 20. The effect of temperature on the release of histamine from normal atrial tissue. Each tissue incubated for 30 minutes in 0.01 N HCl at the temperature shown.


HISTAMINE RELEASED (MOLES per GRAM DRY WEIGHT x 10^{-7})
0
2
4
6
8
10
12
14
16
18
20
20
24
28
32
36
40
44
48
52
TEMPERATURE (°C)

The total releasable histamine of the right and left atria was studied in five experiments, on seven matched atrial pairs per experiment. by sequentially releasing the histamine first in the presence of antigen, and then in the presence of acid. The separated atria, obtained from guinea pigs passively sensitized with antiovalbumin, were first challenged for 10 minutes at 37°C in a 0.1 mg/ml solution of ovalbumin, and then transferred to a solution of 0.01 N HCl at 37°C for a 30 minute acid extraction. The results of the chemical histamine analyses of these two solutions, given in Table VI, show that the right atrium released over 40% more histamine per unit dry weight than did the left atrium, confirming the results of Zilletti, *et al.* (210).

Thus, although the anaphylactic histamine release of the left atrium is only 50% that of the right atrium (Table V), this discrepancy cannot be fully accounted for simply by a difference in the mobilizable stores of the material in the two tissues.

Several experiments were performed to determine if a correspondence could be established between the greater histamine release of the right atrium and the existence in that tissue of the sino-atrial node.

Intra-Atrial Variations in Histamine Release. Right atria were obtained from guinea pigs actively sensitized to ovalbumin. Each right atrium was dissected by a ventral to dorsal cut, circumventing the vena caval orifice in such a way that the region thought to contain the pacemaker remained with the same half each time. The matching

TABLE VI

Comparison of the Total Releasable Histamine of Left and Right Atria*

Number of Hearts	Mean Total Releasable Histamine** Moles/gram dry Tissue ($\times 10^{-7}$)		Ratio Right/Left
	Left	Right	
7	9.10	11.36	1.25
7	9.74	14.13	1.45
7	10.32	16.24	1.57
7	11.95	18.43	1.54
7	11.69	15.14	1.30

Mean ratio: 1.44***

Std. Error: ± 0.09

95% Confidence Interval: 1.27 - 1.63

* All tissue passively sensitized

** "Total releasable histamine" represents the sum of histamine released by anaphylaxis and that subsequently released by acid extraction (0.01 N HCl, 30 minutes, 37°C).

*** All statistics performed on $\log_{10}$ data; antilog data given.

halves of each atrium were then labelled, placed in separate vessels of Chenoweth's solution, and incubated at 37°C for 30 minutes. At this time the tissues were visually checked for mechanical activity; atria were selected for testing if one-half showed strong activity while the other half did not beat at all. Mechanical activity was thus used as evidence that the atrial fragment contained active pacemaker tissue.

These matched, right atrial fragments were then challenged for 10 minutes at 37°C with a 0.1 mg/ml solution of ovalbumin; the tissues were saved for dry weight determinations, and the supernatants assayed for histamine. Table VII-A, which presents data for 8 matched, right atrial fragments, shows that the portion retaining active nodal tissue released 37% more histamine than did the inactive half. On the other hand, Table VII-B shows that there was essentially no difference between the histamine release of left atrial fragments prepared and tested in precisely the same way.

Although this examination of the variations in histamine release of the cardiac tissues emphasized the large differences in the histamine-releasing properties of the right and left atria, it also showed convincingly that atrial tissue was a convenient and reliable source of copious anaphylactic histamine release. Accordingly, the ensuing investigations into the effects of antigen concentration and temperature on the magnitude and rate of this release were made on intact atrial pairs.

TABLE VII

Intra-Atrial Variations in Anaphylactic Histamine Release*

A. RIGHT ATRIUM

FRAGMENT 1		FRAGMENT 2		
Histamine Release (moles/gram dry tissue x 10^{-7})	Mechanical Activity	Histamine Release (moles/gram dry tissue x 10^{-7})	Mechanical Activity	Ratio 1:2
3.07	0	4.19	+	0.73
5.31	0	7.21	+	0.74
3.59	0	4.83	+	0.74
2.06	0	2.81	+	0.73
1.42	0	1.93	+	0.74
2.46	0	3.49	+	0.70
4.87	0	6.60	+	0.74
1.68	0	2.27	+	0.74

Mean ratio = 0.73
Std. error = ± 0.005**

B. LEFT ATRIUM

FRAGMENT 1	FRAGMENT 2	
Histamine Release (moles/gram dry tissue x 10^{-7})	Histamine Release (moles/gram dry tissue x 10^{-7})	Ratio 1:2
2.89	3.60	0.80
3.13	2.44	1.28
3.70	3.42	1.08
4.58	4.59	1.00
4.09	5.39	0.76
2.69	2.43	1.11
3.95	4.90	0.80
1.60	1.86	0.86
3.15	3.01	1.05

Mean ratio = 0.97
Std. error = ± 0.06**

* All tissue actively sensitized to ovalbumin; "anaphylactic histamine release" represents the histamine released by a 10 minute challenge with 0.1 mg/ml ovalbumin at 37°C.

** Calculated by the "range method".

The Effect of Antigen Concentration on Atrial Histamine Release.

Actively Sensitized Tissue. Whole atria, obtained from 8 actively sensitized guinea pigs, were challenged at 37°C for 10 minutes in a solution of ovalbumin at a concentration of 0.001, 0.01, 0.1, or 1.0 mg/ml. Figure 21 shows the average histamine release for the two atrial pairs tested at each concentration (filled triangles).

In Vitro Sensitized Tissue. The second curve of Figure 21 (filled circles) demonstrates the variation of atrial histamine release with antigen, for concentrations covering 5 orders of magnitude. These 18 atria were passively sensitized *in vitro* by incubation for one hour at 37°C in a 100 ug/ml solution of antiovalbumin. Each tissue was then challenged for 10 minutes at the same temperature in one of 18 concentrations of ovalbumin ranging from 0.044 ug/ml to 903 ug/ml.

The Effect of Temperature on Atrial Histamine Release.

The following studies were made for the purpose of determining the shape of the curve of anaphylactic histamine release as a function of temperature. This information was then used to estimate the overall activation energy of the process.

Time-Course of Histamine Release. Preliminary experiments were made to determine the profile of histamine output following challenge at various temperatures so as to set the limits of time necessary for the collection of representative samples. Figure 22 shows the histamine release profiles of two actively sensitized atria, challenged at 27°C

Figure 21. The effect of antigen concentration on the anaphylactic release of histamine from actively sensitized (▲—▲), and *in vitro* sensitized (●—●), guinea pig atria. Challenge temperature, 37°C.

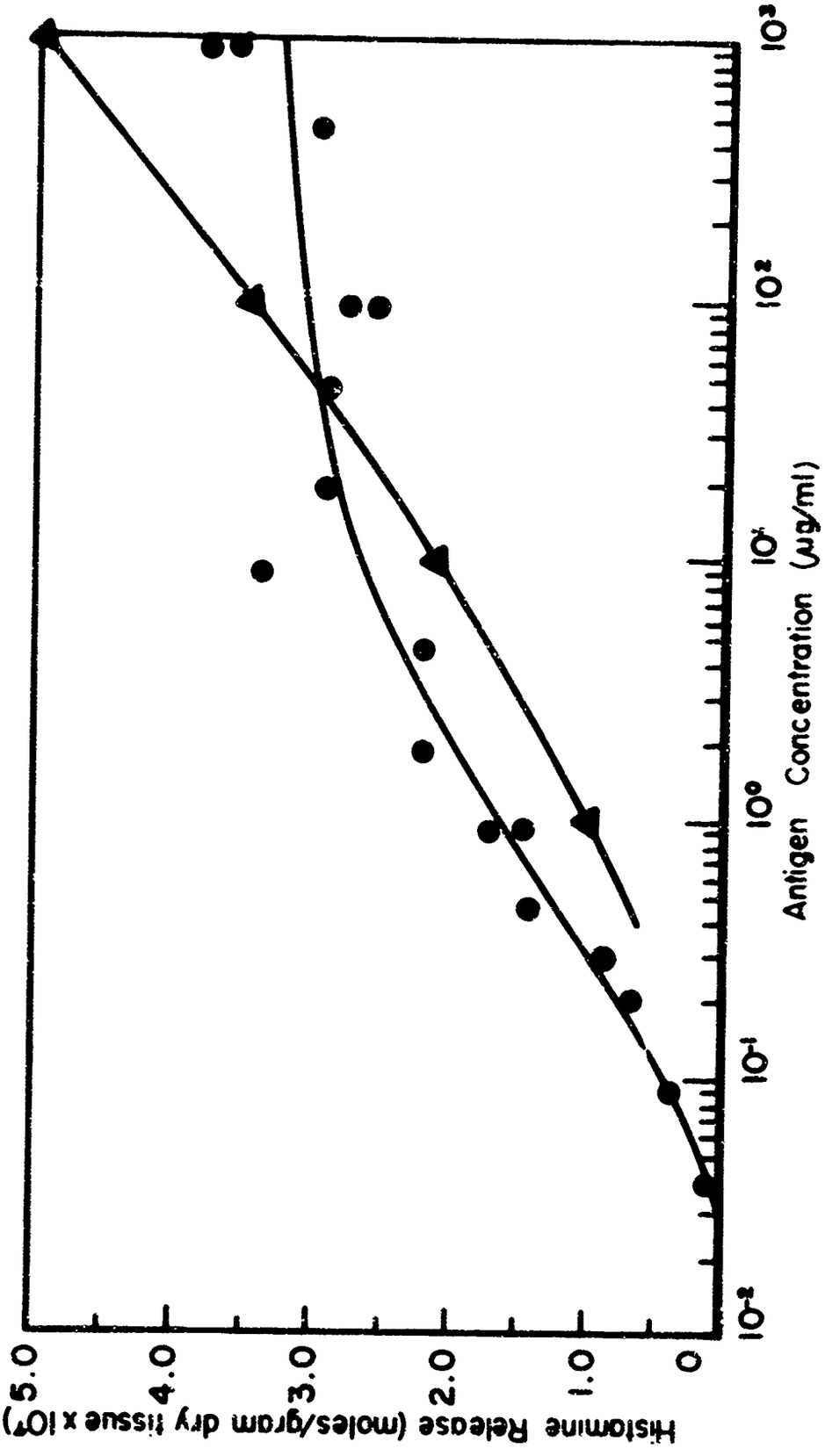
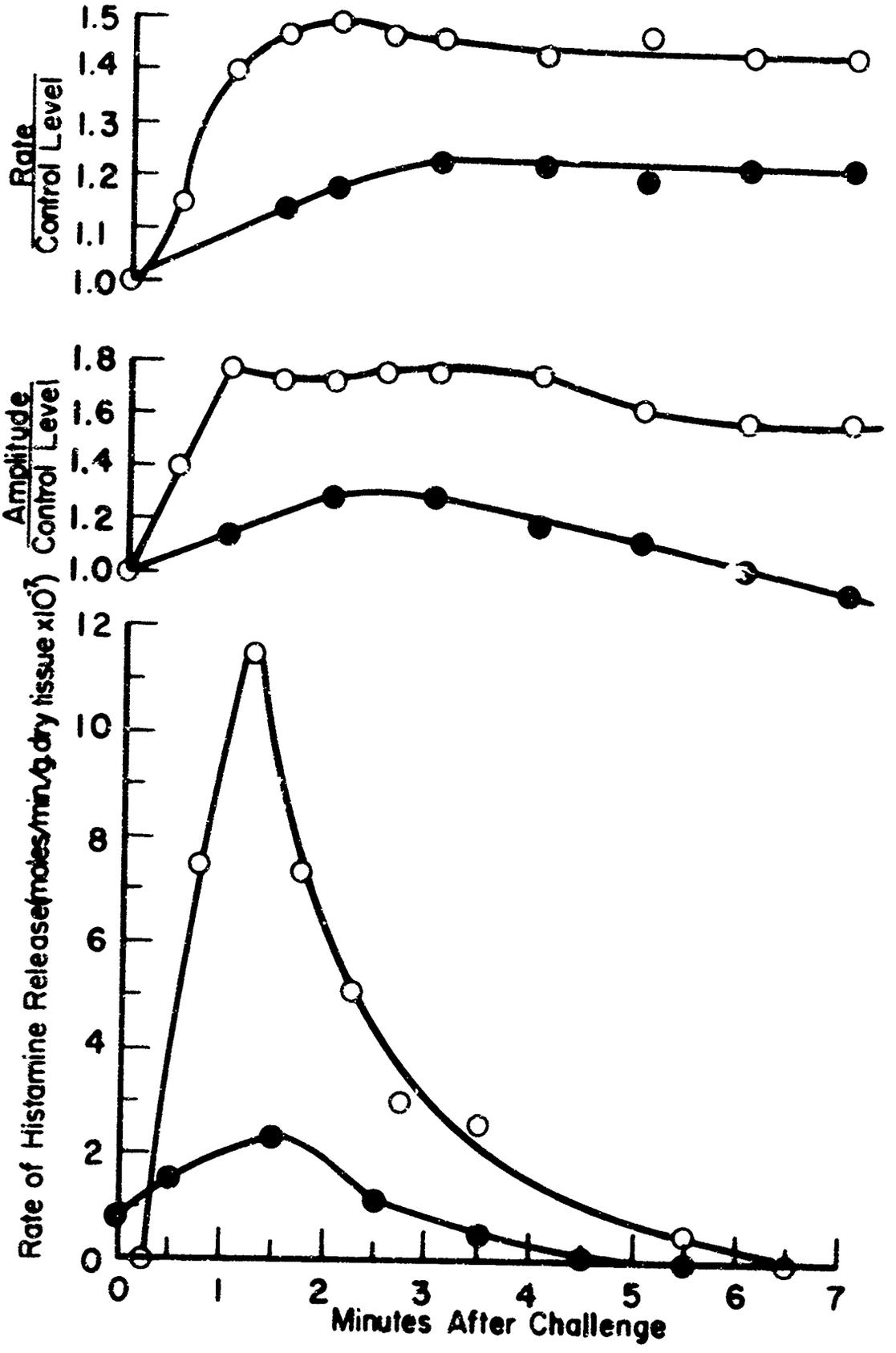

Histamine Release (moles/gram dry tissue x 10⁷)
5.0
4.0
3.0
2.0
1.0
0
10⁻²
10⁻¹
10⁰
10¹
10²
10³
Antigen Concentration (µg/ml)

Figure 22. Time-course of anaphylaxis in isolated, actively sensitized atria: histamine release, and rate and amplitude of contraction as a function of time after challenge at two temperatures (31°C ○—○, 27°C ●—●).

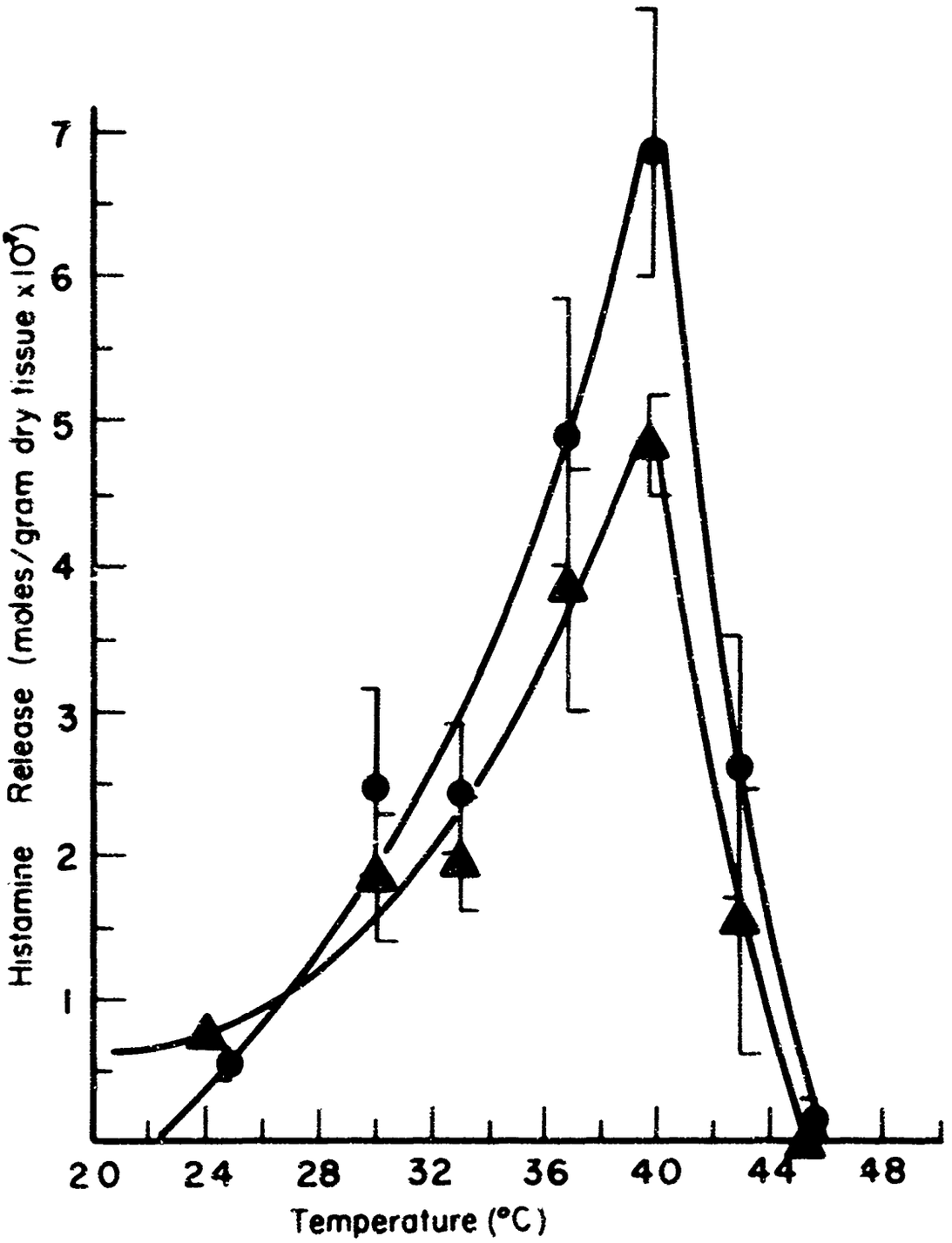

Rate / Control Level
1.5
1.4
1.3
1.2
1.1
1.0
Amplitude / Control Level
1.8
1.6
1.4
1.2
1.0
Rate of Histamine Release (moles/min/g dry tissue x10³)
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
Minutes After Challenge

or 31°C by the superfusion of 0.1% ovalbumin at the rate of 5 ml/minute. In both cases the rate and amplitude of the spontaneously beating atria were increased, the onset of these effects being earlier and the duration longer at the higher temperature. Also, in both cases, the histamine output reached a maximum value 1.5 minutes after the challenge perfusion was begun. At the higher temperature, the total output was about four times as great as it was at 27°C.

Temperature Optimum. The temperature optimum of the anaphylactic release of histamine was studied on atria from the hearts of actively and passively sensitized guinea pigs. The experiment was begun by preconditioning the tissues for 10 minutes in 10 ml of antigen-free medium at 25, 30, 33, 37, 40, or 46°C. Each atrial preparation was then challenged at its respective temperature for 10 minutes in 10 ml of 0.01% ovalbumin.

The effect of temperature on the anaphylactic release of histamine is presented in Figure 23, which gives the means and standard errors of 5 experiments made on actively sensitized, and of 4 experiments on passively sensitized tissues. In both cases the temperature optimum is 39.6°C. Except for the fact that the histamine release at the maximum is about 40% greater in the actively sensitized atria, the temperature curves of the two systems are essentially identical. The rising slopes have an upward concavity and the descending slopes are almost linear and much steeper than the ascending functions. From these experiments, it appears that the effective range for the anaphylactic release of

Figure 23. The effect of temperature on the anaphylactic release of histamine from actively (●—●), and passively (▲—▲) sensitized atria. Incubation (10 min) and challenge (10 min) carried out at temperature shown. Error bars indicate ± 2 x standard error of the mean.

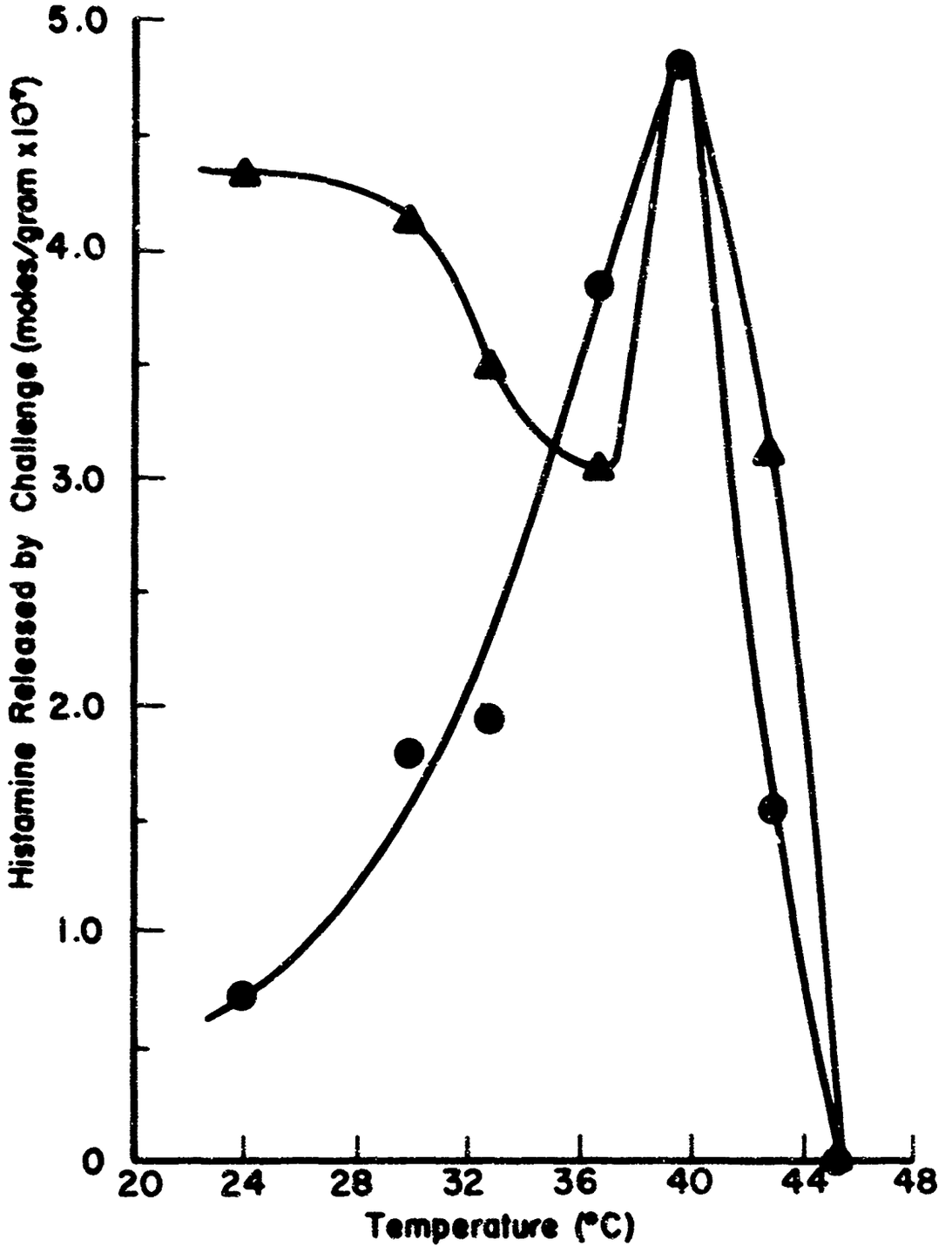

Histamine Release (moles/gram dry tissue x10⁹)
7
6
5
4
3
2
1
20
24
28
32
36
40
44
48
Temperature (°C)

histamine lies between the limits of 24 - 44°C.

The shape of the temperature curves presented in Figure 23 suggests that these functions are actually the outcome of two competing effects of temperature on the processes responsible for histamine release. One effect, that predominates at the lower temperatures, has a positive temperature coefficient; the other effect has a negative coefficient and is responsible for the rapid fall off at higher temperatures. The curves shown in Figure 24 further emphasize this dichotomy, and demonstrate that, while the positive coefficient effect is reversible, the higher temperature effect is not. The lower curve (filled circles), repeated from Figure 23, represents the histamine released by actively sensitized tissues incubated and challenged at the same temperature. The upper curve, however, presents the effects of temperature on the histamine release by tissues incubated at the temperature shown, but challenged at 37°C. The fact that the curves are nearly the same at the higher temperatures indicates that the effect of incubation at these temperatures is not reversed by challenging at a lower temperature (37°C). However, the diminished release from the tissues incubated at the lower temperatures is reversible as evidenced by the augmented output obtained by challenging at 37°C.

Activation Energy. An estimate of the overall activation energy of histamine release was calculated by applying the Arrhenius equation to the rising limb of the temperature function for actively sensitized tissues exhibited in Figure 23.

Figure 24. The effect of temperature on the anaphylactic release of histamine from actively sensitized atria. Incubation and challenge at the temperature shown (●—●); incubation at the temperature shown, challenge at 37°C (▲—▲).


5.0
4.0
3.0
2.0
1.0
0
Histamine Released by Challenge (moles/gram x 10⁶)
20
24
28
32
36
40
44
48
Temperature (°C)

Arrhenius (211) found that the velocity of a chemical reaction could be related to the temperature by

$$\frac{d \ln k}{dT} = \frac{\Delta E_a}{RT^2} . \qquad (1)$$

This equation can be integrated to yield a more useful form relating the activation energy and the rate constants at two temperatures:

$$\log\left[\frac{k_2}{k_1}\right] = \frac{E_a}{2.303\ R}\left[\frac{T_2 - T_1}{T_1 T_2}\right], \qquad (2)$$

where

E_a = activation energy (calories/mole)

R = universal gas constant (1.987 calories/mole/°K)

T_1 = absolute temperature (°K) at which the rate constant, k_1, was measured.

T_2 = absolute temperature (°K) at which the rate constant, k_2, was measured.

The activation energy, E_a, represents the energy that a mole of the reactant must have before a reaction is possible. Graphically, the activation energy is the slope of the line relating the logarithm of the specific reaction rate to the reciprocal of the absolute temperature.

For the present purposes, the magnitude of the histamine release,

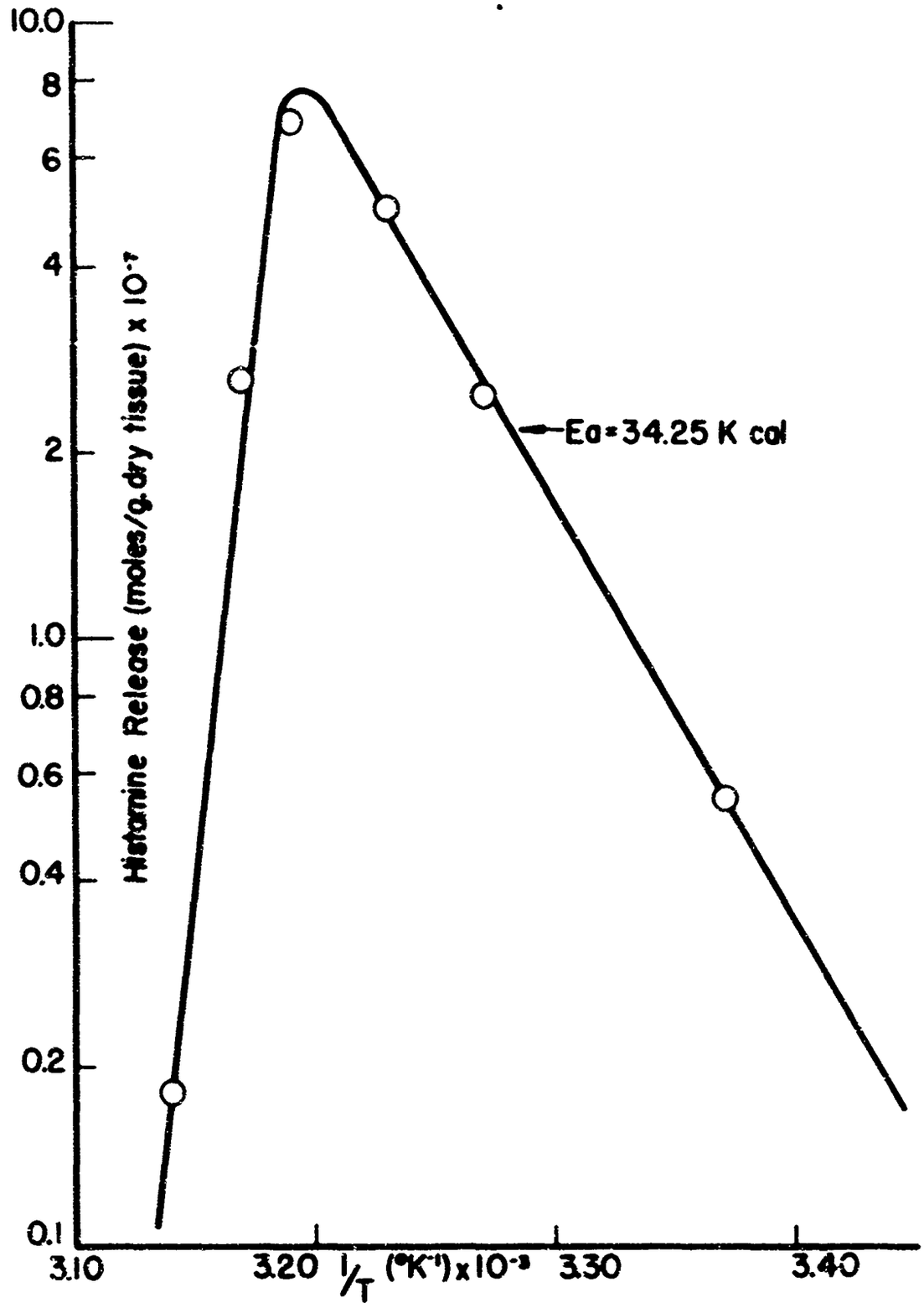
measured over a constant interval of time, can be used as an indication of the reaction rate, and an Arrhenius function can be obtained by plotting the logarithms of the histamine release at various temperatures as a function of the reciprocal of the absolute temperature (Figure 25). When two values of histamine release and their corresponding values of absolute temperature are obtained from the negative-slope portion of Figure 25 and substituted into equation (2), a value of 34.25 kcal/mole is calculated for the overall activation energy of histamine release from atrial tissue.

DISCUSSION

Histamine Release from Cardiac Tissues

The contribution of anaphylactically released histamine by the separate cardiac tissues to the overall release by the whole heart can readily be assessed. According to Table IV, one gram of whole dry heart is composed of 118 mg of atrial, 62 mg of aortic, and 820 mg of ventricular tissue (11.8, 6.2, and 82%, respectively). Multiplying the histamine release for each tissue by its percentage by weight of the whole heart, we obtain the relative contribution of that tissue to anaphylactic release by one gram of dried whole heart. The results of these calculations, seen in Table IV, show that the atria, which comprise only 12% of the dry weight of the heart, contribute over half of the histamine released during cardiac anaphylaxis.

Figure 25. Dependence of the logarithm of histamine release on the reciprocal of absolute temperature. Data of Figure 23. E_a represents the overall activation energy of anaphylactic histamine release from the atria.


10.0
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.1
Histamine Release (moles/g.dry tissue) x 10⁻⁷
Ea=34.25 K cal
3.10
3.20
1/T (°K⁻¹)x10⁻³
3.30
3.40

Histamine Release from Atrial Tissue

The left and right atria, however, differ greatly in their histamine releasing properties. The data of Table V illustrate that when left and right atria from the same heart are sensitized and challenged in an identical manner, the right atrium releases twice as much histamine as does the left atrium. Such a discrepancy between these two similar tissues could arise as a result of differences in (i) degree of sensitization; (ii) one or more of the complicated series of events, triggered by the union of antigen and antibody, which lead to the release of histamine from preformed stores; (iii) the amount of mobilizable material in these stores; or (iv) some combination of these factors.

The experiments summarized in Table VI demonstrate that a part of the greater release of histamine by the right atrium can be accounted for by a larger store of readily releasable material in that tissue. By first releasing histamine from left and right atria anaphylactically, and then by mild treatment with acid, we found that the right atrium contained 40% more histamine in a readily mobilizable form.

However, this does not account entirely for the greater release by the right atrium. From Table VI, we see that

$$L_{total} = 0.70\ R_{total}; \qquad (3)$$

Table V shows that

$$L_{anaph.} = 0.50\ R_{anaph.}, \qquad (4)$$

in which L_{total} and R_{total} represent the total releasable histamine, and $L_{anaph.}$ and $R_{anaph.}$, the anaphylactically releasable material, of the left and right atrium, respectively. Dividing equation (4) by equation (3), we obtain that

$$\frac{L_{anaph.}}{L_{total}} = \frac{0.50}{0.70} \frac{R_{anaph.}}{R_{total}}, \qquad (5)$$

or

$$\frac{L_{anaph.}}{L_{total}} = 0.71 \frac{R_{anaph.}}{R_{total}}, \qquad (6)$$

which states that, even if we correct for the difference in the amount of histamine available for release by expressing the anaphylactic release per unit of total releasable histamine, the right atrium still releases 40% more histamine than does the left atrium. *The right atrium not only contains a larger store of mobilizable histamine, but it releases a greater percentage of this store when challenged with specific antigen.*

That at least part of the greater anaphylactic histamine release of the right atrium is due to the sino-atrial node in that tissue is shown by the data of Table VII. When right atria, from actively

sensitized guinea pigs, were divided into two segments, the segment exhibiting mechanical activity released 37% more histamine when challenged with specific antigen, than the segment showing no activity. If mechanical activity in an atrial fragment indicates the presence of viable, active nodal tissue, then the above result is circumstantial evidence that this nodal tissue is, at least partly responsible for the greater histamine release of the intact right atrium, compared to that of the left atrium.

Factors Affecting Atrial Histamine Release

Antigen Concentration. The concentration of antigen used to challenge sensitized tissue is a determinant of the amount of histamine subsequently released. Figure 21 shows this to be true both for actively sensitized and *in vitro* sensitized guinea pig atria. These results demonstrate, in both instances, an increase of histamine output with increasing antigen concentration and indicate that, at the levels of antigen employed, neither system was subjected to antigen concentrations high enough to inhibit histamine release. Analysis of the reaction solutions showed that detectable amounts of histamine were released from *in vitro* sensitized atria by concentrations of ovalbumin as low as 0.086 ug/ml, and that maximum release occurred between concentrations of 20 and 100 ug/ml of antigen.

Time-Course of Histamine Release. Our studies illustrate the explosive nature of the anaphylactic release of histamine from atrial

tissue. This release obtained its maximum rate at 1.5 minutes following challenge, and was virtually complete within 7 minutes (Figure 22). Therefore, a 10 minute challenge reaction was considered to be sufficient to provide for complete recovery of the released histamine with a minimum of enzymatic destruction. The general shape of this release profile and the time of maximum rate of release are in good agreement with the results reported by Feigen, *et al.* (212) for the release of histamine from sensitized whole hearts.

The upper curves of Figure 22, those showing the changes in rate and amplitude of contraction following challenge, show a time-course which corresponds closely with that of the release curve. This lends support to the accepted notion that the mechanical alterations subsequent to anaphylaxis in the heart are a result of the release of histamine from cardiac tissues.

Effect of Temperature. The curves of histamine release as a function of incubation-challenge temperature (Figure 23) confirm the results of Mongar and Schild (187), who found that the release of histamine from guinea pig lung was zero at 15°C, rose to a maximum between 40 and 41°C, and fell abruptly to zero again at 44 to 45°C. These functions, described by a gradual, monotonic activation at the lower temperatures, and a rapid inactivation at temperatures above the optimum, resemble the rate curve of an enzyme reaction. The rate of such a reaction at a given temperature is the resultant of two competing processes: (i) the increase in reaction rate with increasing temperature

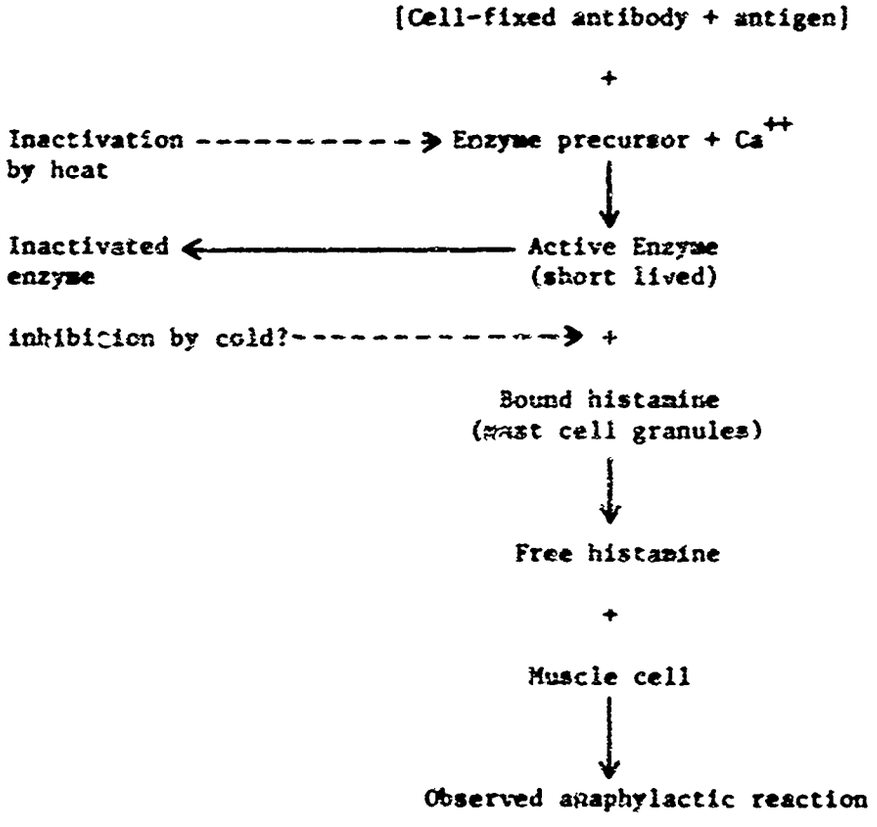
due to the additional kinetic energy of the molecules of the reactants; and (ii) the decrease in reaction rate, above a certain temperature, due to denaturation of the protein enzymes.

Our results (Figure 23) show that incubating atrial tissue for 10 minutes at 43°C reduces the subsequent histamine release at that temperature to 40% of maximum, while a 10 minute incubation at 45°C completely abolishes histamine release. Figure 24 demonstrates that this heat inactivation is not readily reversible: challenging the tissues at 37°C after the 10 minute incubation at 43 or 45°C does not increase the histamine output. Mongar and Schild (187) found that sensitized guinea pig uterus, incubated at 45°C for 5 minutes and then challenged at 37°C, failed to give a Schultz-Dale reaction, but responded to histamine. Similarly, sensitized guinea pig lung failed to release histamine in response to antigen after a 5 minutes incubation at 45°C, although its oxygen consumption was unchanged.

These authors postulated that the anaphylactic reaction depends on a heat-labile factor, and proceeded to study the nature of this factor by the technique of passive *in vitro* sensitization. When they sensitized lung tissue with antibody which had been preheated to 45°C, they observed normal sensitization; however, when normal antibody was incubated with lung tissue previously heated to 45°C, no sensitization occurred. These results demonstrated that the putative heat-labile factor was not the antibody, but a constituent of normal tissue, and supported the idea that this factor was an enzyme or enzyme

precursor required for the anaphylactic mechanism.

This idea of a heat labile enzyme is an integral part of a general scheme for the anaphylactic release of histamine, proposed by Mongar and Schild (139). This scheme is based on the theory that the union of antigen with cell-bound antibody results in the activation of an enzyme system, required for histamine release, which is short-lived and rapidly inactivated. The active enzyme, which acts in some way to release histamine, is formed by the action of the antigen-antibody reaction on the inactive enzyme precursor, which is the heat labile factor postulated above. Mongar and Schild (139) represented this scheme diagramatically as follows:



The mechanism of inhibition of anaphylaxis by cooling is different from that of warming in that cooling has no permanent effect (187). A sensitized tissue which is cooled and then returned to 37°C responds normally to antigen (Figure 24). Although the tissues incubated and challenged at temperatures below 37°C show decreased histamine output, the tissues incubated at these lower temperatures, but challenged at 37°C, show a histamine release equal to, or higher than, that of tissues incubated and challenged at 37°C. Mongar and Schild (187) found that at 17°C, histamine release from sensitized lung was completely inhibited, but desensitization was not completely inhibited. When the tissue was rewarmed to 37°C, a reduced histamine release occurred due to the partial desensitization. In terms of their hypothesis of temporary activation of an enzyme system, this effect of low temperature could be explained. At 17°C, the rate of formation and destruction of the enzyme was diminished, but not completely inhibited, while the action of the enzyme was completely prevented. This resulted in a complete inhibition of histamine release at that temperature and a diminished release after rewarming, since, by then, some of the activated enzyme had been inactivated. This proposal would place the location of inhibition by cold at some later stage in the reaction scheme shown above.

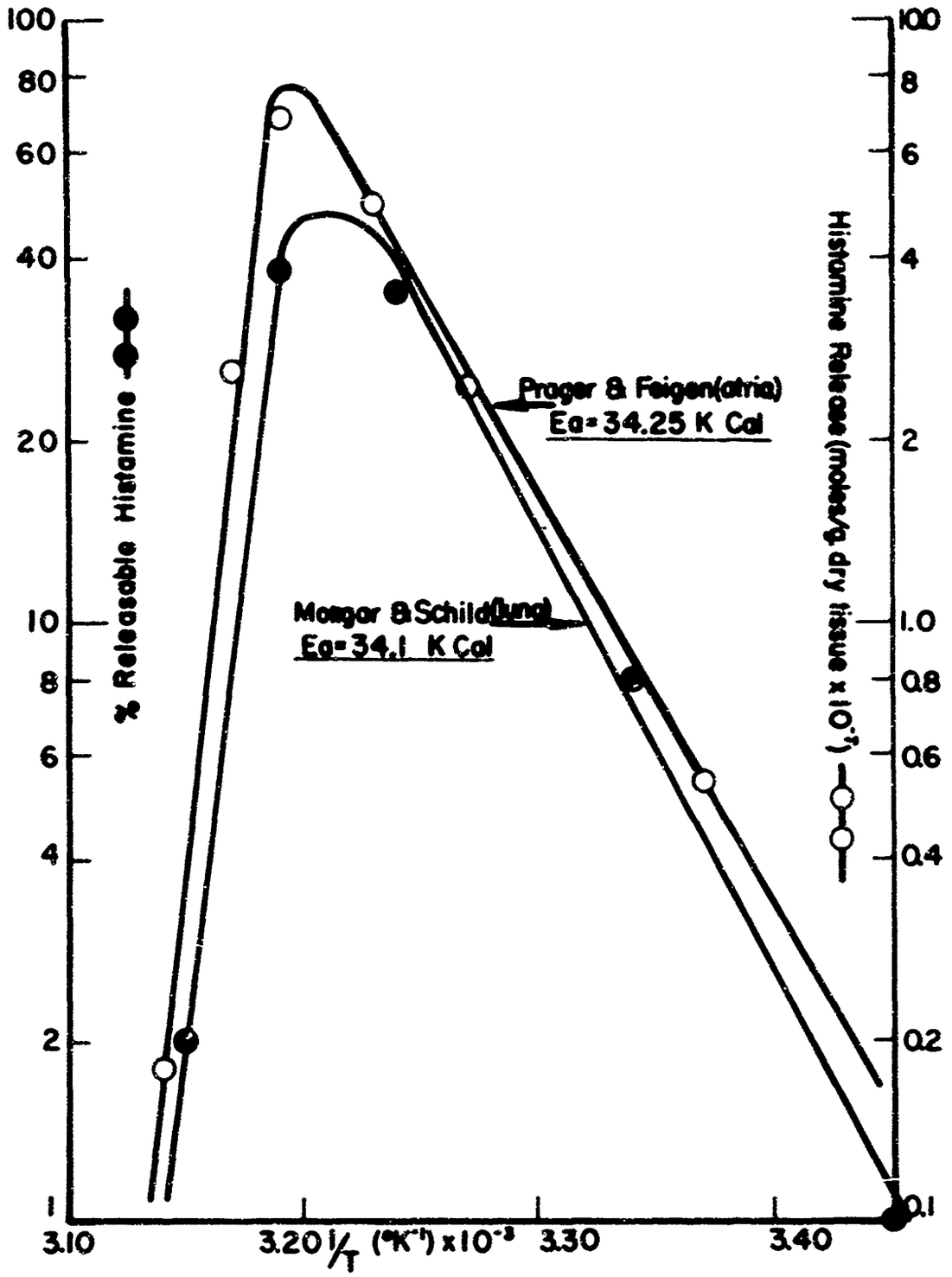
Activation Energy. The activation energy can be calculated from the increase of histamine release by antigen with increased temperature. Figure 25 shows an Arrhenius plot of the data presented in Figure 23 for the release of histamine by actively sensitized atrial tissue at various temperatures. Plotting the logarithm of histamine release as

a function of the reciprocal of the absolute temperature according to the form of the Arrhenius equation, linearizes the activation curve (now on the right in Figure 25) and the inactivation curve. By selecting two values of temperature and their corresponding values of histamine release from the activation side of Figure 25, and substituting them into the Arrhenius equation [equation (2)], we calculate an activation energy for the anaphylactic release of histamine from atrial tissue of 34.25 kcal.

This can be compared with the accepted value of 45 kcal calculated by Mongar and Schild (187) for histamine release from guinea pig lung. However, these authors obtained this value for the activation energy by first determining the Q_{10} of activation, and then substituting their value of the Q_{10} (nearly 12) into the Arrhenius equation. The Q_{10} of a non-linear function is a gross estimate of the energies involved. It is far more accurate to transform the data into the form of the Arrhenius plot and use the Arrhenius equation directly. If the data which Mongar and Schild used for their Q_{10} calculation (187) are replotted according to this method, we obtain a curve which closely resembles our Arrhenius plot of atrial histamine release (Figure 26). From this function we calculate an activation energy for the histamine release from lung tissue of 34.1 kcal which is virtually identical with the 34.25 kcal activation energy for atrial histamine release.

The Arrhenius equation can also be applied to the left-hand portion of the curves of Figure 26 in order to calculate a value for the energy of inactivation of histamine release. When this is done, a value of

Figure 26. Dependence of the logarithm of histamine release on the reciprocal of absolute temperature. A comparison of two values for the overall activation energy (E_a) of anaphylactic histamine release.


100
80
60
40
20
10
8
6
4
2
1
% Releasable Histamine
Prager & Feigen(atria)
Ea=34.25 K Cal
Mongar & Schild(lung)
Ea=34.1 K Cal
100
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.1
Histamine Release (moles/g dry tissue x 10⁻⁹)
3.10
3.20
3.30
3.40
1/T (°K⁻¹) x10⁻³

about 190 kcal is obtained. This is of the same order as inactivation energies of protein denaturation found for egg albumin, 136 kcal (213), and goat serum complement, 199 kcal (214), and other proteins (215).

The value of 34 kcal obtained for anaphylactic histamine release is likely to include, in addition to the activation energy of the enzymatic histamine releasing system on subcellular particles, the activation energies of antigen-antibody reactions, and, perhaps, the activation energy of sensitization. In an attempt to determine specifically the activation energy of histamine release, alone, Vurek, *et al.* (153) measured the initial velocity of histamine release from *in vitro* sensitized guinea pig atria at 3 temperatures, under conditions in which only the challenge step was a variable of temperature. They felt that this approach was justified in light of the general schema proposed by Mongar and Schild (139), in which the immediate consequence of the antigen-antibody reaction on the cell surface is the activation of a short-lived thermolabile enzyme which releases histamine from its bound form. If, then, under controlled conditions, histamine release is taken as an index of the magnitude of anaphylaxis, the velocity of the output should be proportional to the number of sites on subcellular particles reacting with the enzyme per unit time; hence, the variation in the initial release velocity with temperature ought to provide a reasonable measure of the activation energy. Accordingly, using this technique, Vurek, *et al.* arrived at a value of 23.2 kcal as an estimate of the activation energy for the release of histamine,

considerably lower than the value of 34 kcal obtained for the overall process.

It is interesting to speculate on what factors account for the 11 kcal difference between the value of 34.2 kcal for the overall process of anaphylactic histamine release, and the value of 23.2 kcal for the actual release of histamine. It may be significant that Feigen *et al.* (154), using the method of passive *in vitro* sensitization, found a value of about 12 kcal for the activation energy of sensitization.

PART III. Contribution of Anaphylaxis to the Overall Cardiotoxicity of Streptolysin O

INTRODUCTION

The main thesis underlying the experimental studies which form the basis of this work has been that the cardiotoxic action of streptolysin O may be aggravated by the presence of specific antibodies in the heart. Part I of this work has established the nature and site of the defect in the normal heart insulted with active toxin. This showed that the early, reversible, physiological changes can be quantitatively accounted for by the release of acetylcholine, whereas the later irreversible changes, occurred because of a defect in the atrio-ventricular conduction system.

In Part II there were examined the rules of quantitative cardiac anaphylaxis in the guinea pig showing that the process was temperature-dependent and that it was owed in great part to the release of histamine. The richest source of histamine was found in the right atrium and, by the method of residue, in the region of the sino-atrial node. Thus Part II showed that the atrium was a reliable test tissue for the subsequent investigation of the possible anaphylactic properties of the toxin.

Based on the fact that the toxin liberates acetylcholine, *sui generis*, and that the anaphylactic reaction liberates histamine, the hypothesis was made that challenge of sensitized hearts by active toxin should result in the production of both materials. In the experiments which follow it will be established that the usual manifestations of cardiac anaphylaxis -- increased rate, amplitude, and histamine release -- obtain in actively and passively sensitized tissues when they are challenged with the

inactive (oxidized) form of the toxin. On the other hand, when tissues sensitized in this way are challenged with the *active* (reduced) form of streptolysin O there is evidence that the response is biphasic; *i.e.* there appears first a reduction in force and rate of contraction, characteristic of the effect of acetylcholine seen on "normal" atria, which is then followed by the typical histamine-like response observed with the inactive toxin and other non-toxic protein antigens. By appropriate use of atropine and pyribenzamine it is shown that either, or both, of the responses can be selectively abolished.

MATERIALS AND METHODS

Immunological Reagents

Antigen:Oxidized Streptolysin O. A stock, 1.0 mg/ml solution of oxidized (inactive) streptolysin O was prepared by dissolving the lyophilized group C material in the standard phosphate buffer. This solution was used as the sensitizing antigen in immunizing rabbits for the production of specific antisera, and in actively sensitizing guinea pigs as a source of sensitized tissues. This same solution was used as the specific challenging antigen in those experiments involving the anaphylactic response of sensitized tissues to oxidized streptolysin O.

Antigen:Reduced Streptolysin O. Solutions of 1.0 mg/ml of reduced (active) streptolysin O were prepared daily by dissolving the lyophilized group C material in phosphate buffer and activating with

cysteine in the usual manner. This solution was used as the specific challenging antigen in studies involving the anaphylactic response of sensitized tissues to reduced streptolysin O.

Antibody. Each of two rabbits was immunized by the subcutaneous injection of 5 mg of oxidized streptolysin O. The toxin, dissolved in equal amounts of buffer and complete Freund's Adjuvant (CFA, Difco Laboratories, Detroit, Michigan), was injected as single subcutaneous depots on either side of the neck. Fourteen days, and twenty-eight days following this initial injection, the two rabbits were given "booster" injections of 5 mg of the oxidized material in CFA, administered intradermally.

At regular intervals, the rabbits were bled by cardiac puncture; the serum was prepared, the γ-globulin fraction obtained, and the antibody titer of these preparations determined, by the techniques presented in the "Materials and Methods" section of Part II. The serum was dialyzed for 3 days with 3 changes of 1% NaCl. The γ-globulin fraction was dialyzed 3 days with 3 changes of borate-buffered saline, pH 8.45, 0.1 μ.

Preliminary characterization of the anti-streptolysin O sera was made by immunoelectrophoresis, and ultracentrifugal analyses. Immunoelectrophoresis was performed in 1% barbital agar, pH 8.2, 0.05 μ; 25 volts was applied across each 10 cm slide for 2.5 hours. The sedimentation coefficient at 20°C, the S_{20} (216,217), was determined

by centrifugation at 52,640 rpm (201,366 x g) for 2 hours in the Beckman Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, California).

Results are presented for the antibody from only one of the two rabbits immunized (rabbit no. 10); antibody from the second rabbit had a lower titer, but was otherwise identical to that of rabbit no. 10.

Experimental Animals

Normal, male, variecolored guinea pigs, ranging in weight from 250 to 450 grams, were used in these studies. Two, male, New Zealand Albino rabbits, initially weighing about 1.8 kg each, were used as the source of anti-streptolysin O antibody.

Preparation of Tissue

Isolated whole hearts, atria, and ileal strips, obtained from normal and sensitized guinea pigs, were prepared, equilibrated, and maintained as described in the "Materials and Methods" section of Parts I and II.

Sensitization of Tissues

Active Sensitization. Each of 12 guinea pigs was immunized by the subcutaneous injection of 1.0 mg of oxidized streptolysin O. The toxin, dissolved in equal amounts of buffer and CFA, was injected as single subcutaneous depots on either side of the neck. These animals, which were used 30 days following immunization, were the source of

actively sensitized whole hearts, atria, and ileal strips, subsequently tested *in vitro* for their anaphylactic responsiveness.

Passive *In Vitro* Sensitization. Whole hearts, obtained from normal guinea pigs, were sensitized *in vitro* by the passage of 1.0 ml of the dialyzed serum or γ-globulin through the coronary circulation at 37°C (70). The perfusate was collected and recycled four times, followed by perfusion with antibody-free Chenoweth's solution for 10 minutes. After this procedure, such hearts were either left in place for those experiments dealing with the anaphylactic response of the whole organ, or removed and appropriately dissected for the anaphylactic studies of the isolated atria.

Challenge

Whole Hearts. Sensitized whole hearts were challenged by the administration of 0.1 mg of oxidized or reduced streptolysin O into the main standpipe.

Atria and Ileal Strips. Sensitized atria and ileal strips, mounted in 3.5 ml muscle baths as described earlier, were challenged by administration of 0.1 mg of oxidized or reduced toxin into the muscle bath (final concentration, 28.6 μg/ml).

All work was performed at 37°C.

Estimation of Histamine

Chemical analyses of challenge solutions were made according to the techniques described in the "Materials and Methods" of Part II.

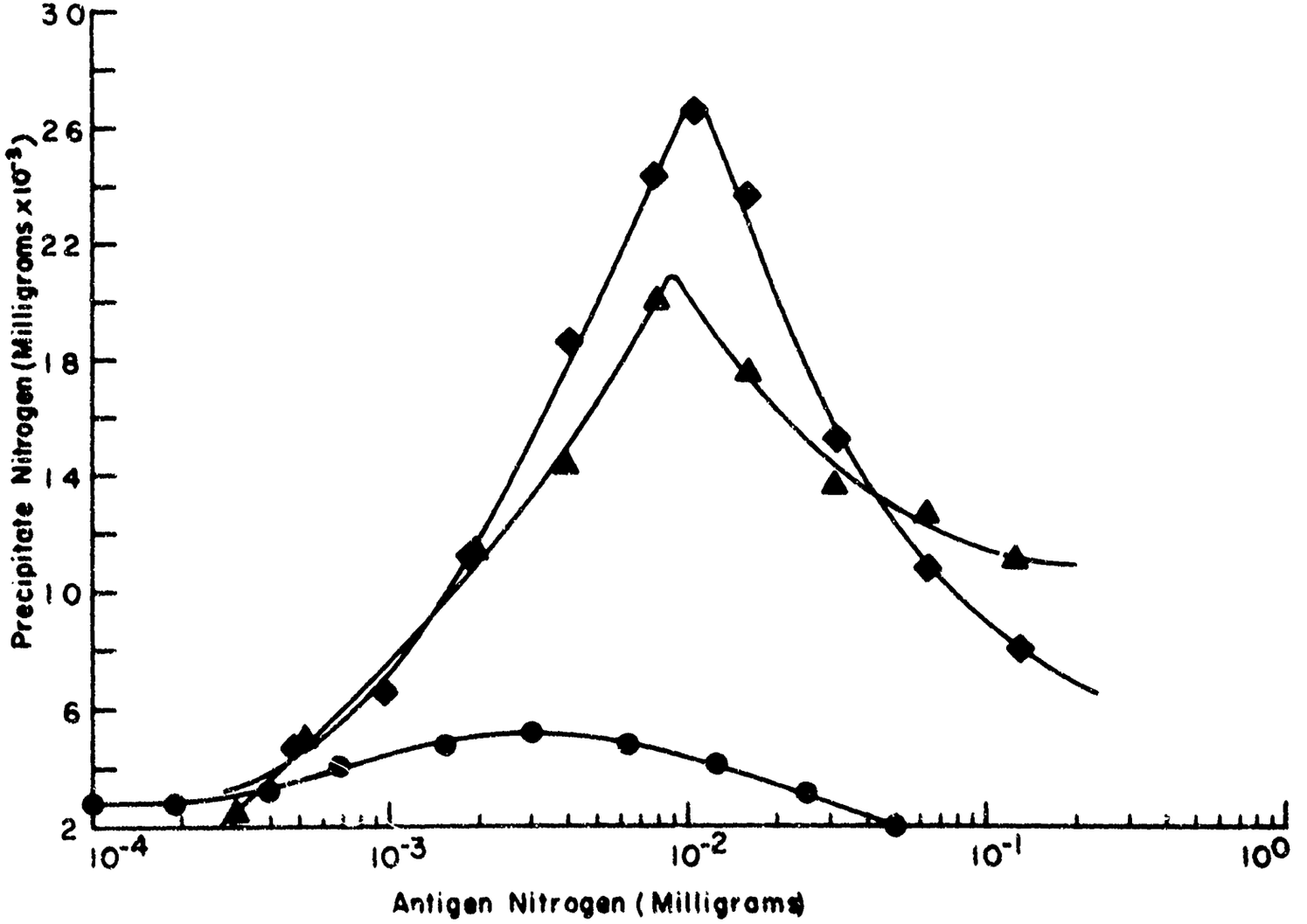
Some challenge solutions were bioassayed on the guinea pig ileum standardized with histamine (see "Materials and Methods, Part I). Histamine specificity was demonstrated by blocking the ileal strips with pyribenzamine (PBZ, Ciba, Basle, Switzerland).

RESULTS

Characterization of Rabbit Anti-Streptolysin O Antibody.

Antibody Titer. Fourteen days following the initial immunization, serum obtained from rabbit no. 10 was found to have a very low specific antibody content. The bottom curve of Figure 27 shows the amount of total precipitable nitrogen obtained when a constant amount of serum was reacted with varying amounts of antigen. The peak of this quantitative precipitin curve represents the reaction mixture containing the optimal proportion (OP) of antigen to antibody. To the left of this point (antibody excess), and to the right of this point (antigen excess), the proportions are not optimal and there is less precipitation. By subtracting the antigen-nitrogen from the total precipitate nitrogen at the OP point, the amount of precipitated antibody can be calculated. By this technique, it was found that the rabbit serum contained only 14 μg/ml specific antibody nitrogen.

Figure 27. Quantitative precipitin curves for 3 collections of rabbit anti-streptolysin O sera: 14 days after initial immunization (●——●); 14 days (▲——▲) and 21 days (◆——◆), after the second "boosting" injection.

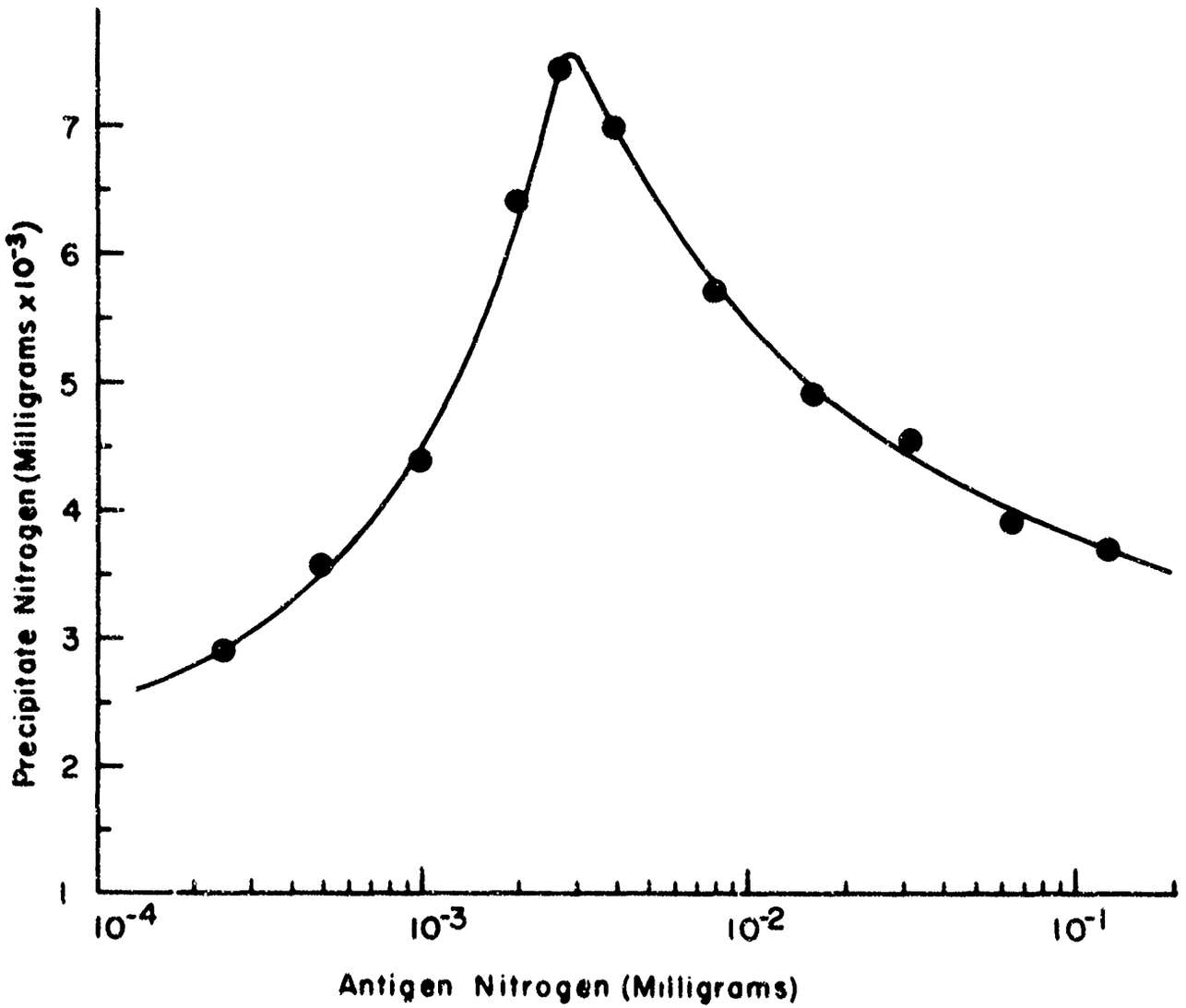

Precipitate Nitrogen (Milligrams x 10⁻³)
30
26
22
18
14
10
6
2
10⁻⁴
10⁻³
10⁻²
10⁻¹
10⁰
Antigen Nitrogen (Milligrams)

In order to increase the antibody titer of the serum, the rabbit was given two intradermal booster injections separated by two weeks. Fourteen days, and twenty-one days, following the second booster injection, the rabbit was bled, and the antibody content of the serum determined. The two upper curves of Figure 27 show that the antibody titer of the serum was indeed improved by this technique. Fourteen days after boosting the serum had a specific antibody content of 92.8 ug antibody nitrogen/ml; after 21 days it had risen to 128 ug antibody nitrogen/ml.

The γ-globulin fraction was precipitated from the latter serum. Although the serum exhibited a relatively high antibody content, the γ-globulin had a specific antibody titer of only 37.6 ug antibody nitrogen/ml (Figure 28). A check of the supernatant of the serum fractionation procedure showed that there was considerable antibody that had not precipitated under the conditions used.

Immunoelectrophoretic Studies. Electrophoresis was performed on the sera obtained at each bleeding. The results of these preliminary tests closely paralleled the titration studies. The early sera showed only very weak, single lines of precipitation with the antigen. After boosting, however, these primary component lines became prominent, and several other components became evident. At the last bleeding, one major, and four minor antigenic components were present.

Figure 28. Quantitative precipitin curve for rabbit anti-streptolysin O γ-globulin.


Precipitate Nitrogen (Milligrams x10⁻³)
7
6
5
4
3
2
1
10⁻⁴
10⁻³
10⁻²
10⁻¹
Antigen Nitrogen (Milligrams)

Analytical Ultracentrifugation. The analytical ultrcentrifuge was used to determine the sedimentation coefficient (S_{20}) of the sera and γ-globulin fraction. These preliminary studies showed that the rabbit serum contained two main sedimentation peaks, one with a value of 13.7*S*, one with a value of 5.6*S*. Centrifugation of the γ-globulin fraction showed only one peak, a 6.8*S* component. In conformance with the observation that there was antibody activity present in the supernatant of the serum fractionation, it was found that this supernatant contained a 19*S* macroglobulin.

Anaphylaxis: Actively Sensitized Tissues

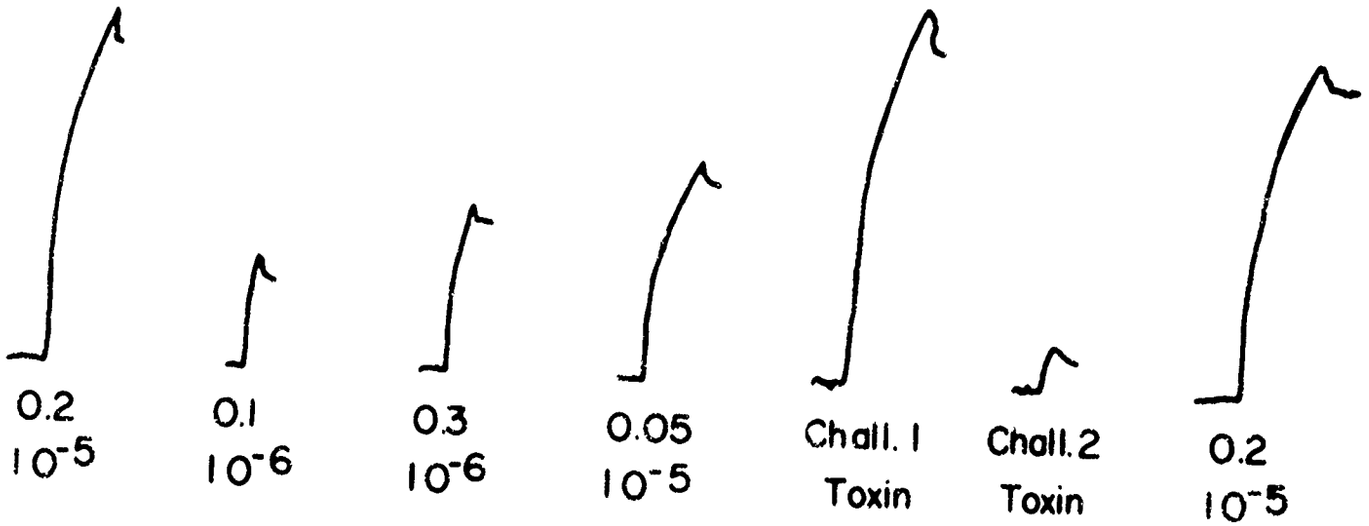
Schultz-Dale on the Guinea Pig Ileum. The ileum was removed from a guinea pig actively sensitized to oxidized streptolysin O, and an ileal strip prepared and mounted in a 3.5 ml muscle bath as described earlier. The ileal strip was first standardized with several concentrations of histamine, and then challenged with 0.1 mg of the oxidized toxin. Figure 29A shows that, whereas this first challenge with antigen produced a rapid, maximal contraction, a second challenge with the same dose of antigen resulted in a very slight contraction. Apparently the first challenge had desensitized the tissue, since the strip was still responsive to histamine.

Figure 29B demonstrates that the response of the sensitized ileum to the first challenge with antigen was indeed a *specific* reaction. When an ileal strip from a normal guinea pig was challenged with the same dose of antigen, it responded with a minimal contraction.

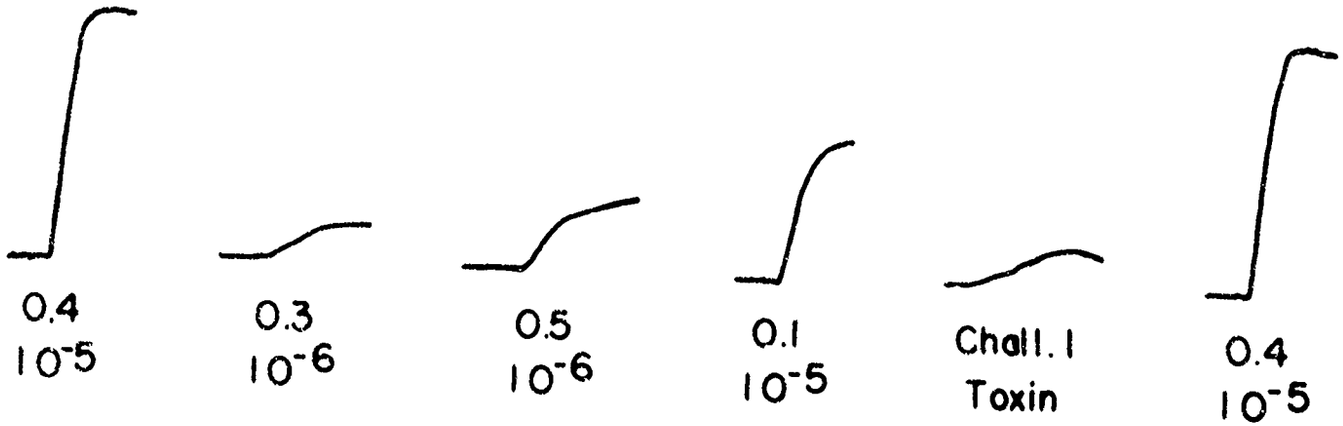
Figure 29. A. Schultz-Dale on the guinea pig ileum actively sensitized to oxidized streptolysin O. Standardization of the ileum with 4 concentrations of histamine was followed by two challenges with antigen (0.1 mg oxidized toxin). The ileum was checked for its responsiveness to histamine.

B. Control. Challenging a strip of normal guinea pig ileum with the antigen (0.1 mg oxidized toxin) resulted in only a slight contraction. Bath volume 3.5 ml, temperature, 37°C.

A. Sensitized Ileum, Oxid. Toxin



B. Normal Ileum, Oxid. Toxin

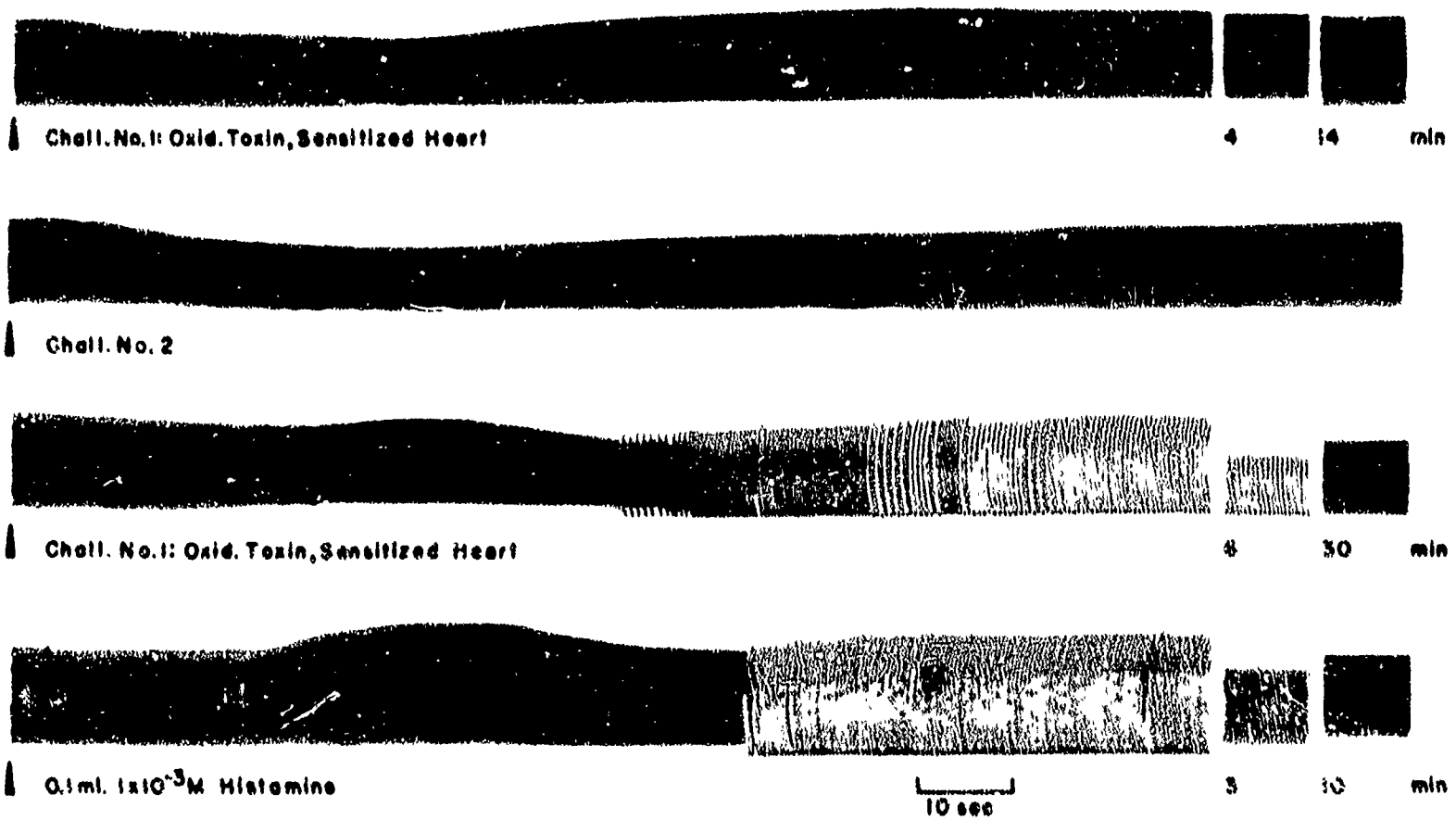


Perfused Whole Heart. The heart from the same actively sensitized guinea pig used above was removed and mounted on the Anderson apparatus in the usual way. After thorough equilibration, the heart was challenged by the administration of 0.1 mg of oxidized streptolysin O to the standpipe above the heart. The first trace of Figure 30 shows a mild, but typical anaphylactic response. The initial depression, a direct effect of the oxidized toxin, was followed by an increase in rate and amplitude, with the rate reaching a value of 330 beats/minute compared with the control rate of 230 beats/minute. The perfusate collected during the height of this anaphylactic reaction, when chemically analyzed for histamine, was shown to contain more than 100 times the control levels of histamine (1.0×10^{-7} moles/minute/gram dry heart, compared to 0.72×10^{-9} moles/minute/gram dry heart in the control perfusate).

The second trace of Figure 30 shows that the heart was almost completely desensitized by the first challenge. The direct effect of the inactive toxin was followed by a gradual recovery to control levels, and the perfusate contained control levels of histamine.

A more severe anaphylactic response was elicited from a second actively sensitized heart challenged with the standard dose of antigen (Figure 30, third trace). The initial depression was interrupted by an increase in rate and amplitude, followed by partial atrio-ventricular block. Such a block is typical of strong anaphylactic responses in the

Figure 30. The response of two whole hearts from guinea pigs actively sensitized with oxidized streptolysin O, to challenge with the specific antigen. Comparison between the first and second challenges on the same heart (traces 1 and 2), and between the anaphylactic response, and the response to authentic histamine (traces 3 and 4). 37°C.

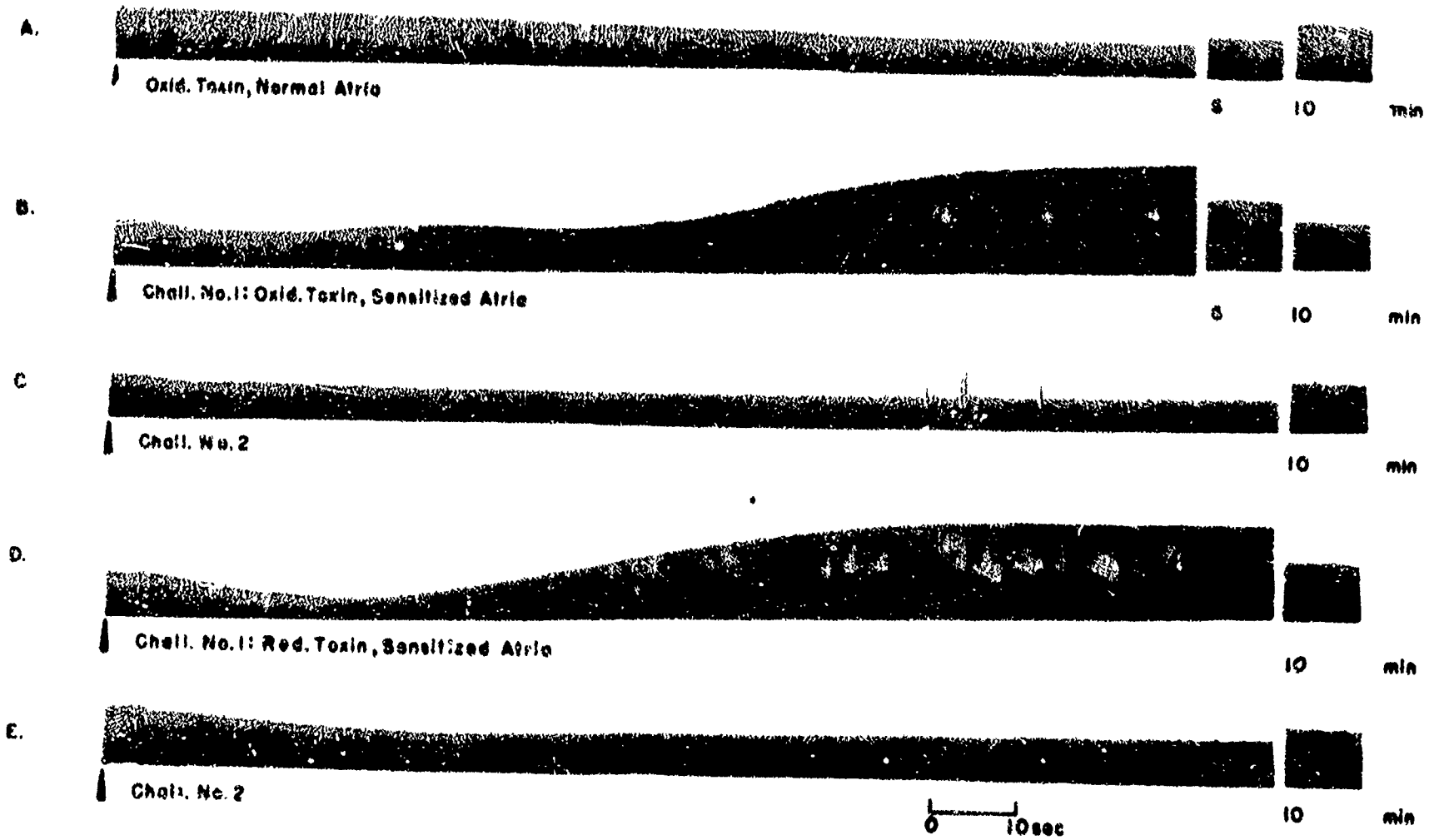

Chall. No. 1: Oxid. Toxin, Sensitized Heart
4
14
min
Chall. No. 2
Chall. No. 1: Oxid. Toxin, Sensitized Heart
4
30
min
0.1 ml. 1x10⁻³M Histamine
10 sec
3
10
min

whole heart, and of the response of the whole heart to histamine. The bottom trace in Fig re 30 shows that this entire anaphylactic response can be reproduced completely by a suitable dose of histamine (70).

Isolated Atria. Atria, obtained from whole hearts of guinea pigs actively sensitized to the oxidized toxin, were prepared and mounted in a 3.5 ml muscle bath in the usual way. When challenged with either the oxidized or reduced toxin, these sensitized atria exhibited strong anaphylactic reactions (Figure 31). Trace A of this figure shows the reaction of a normal, unsensitized atrial preparation to challenge with the antigen (oxidized streptolysin O, 0.1 mg). When the same dose of the same antigen was added to the bath surrounding a sensitized atrial pair, the tissue responded with an extreme increase in the rate and amplitude of contraction (Trace B). A second challenge with antigen (Trace C) produced none of these effects. Chemical analyses of the baths collected at 3 minutes post-challenge showed that the first challenge released 2.45×10^{-7} moles of histamine/gram dry atria, compared to 0.26×10^{-7} moles released by the second challenge; both the pre-challenge bath solution and the challenge solution from the normal tissue (Trace A) were negative for histamine.

Similar results were obtained when sensitized atria were challenged with reduced streptolysin O (Traces D and E). Except for a much more pronounced initial depression due to the direct effect of the active toxin, the anaphylactic response to this antigen was identical to that

Figure 31. The anaphylactic response of isolated atria from guinea pigs actively sensitized with oxidized streptolysin O, to challenge with the oxidized toxin (Traces B and C) and the reduced toxin (Traces D and E). Standard challenge dose of 0.1 mg of antigen added to 3.4 ml of Chenoweth's solution. 37°C.


A.
Oxid. Toxin, Normal Atria
5
10
min
B.
Chall. No.1: Oxid. Toxin, Sensitized Atria
5
10
min
C
Chall. No. 2
10
min
D.
Chall. No.1: Red. Toxin, Sensitized Atria
10
min
E.
Chall. No. 2
10
min
0
10sec

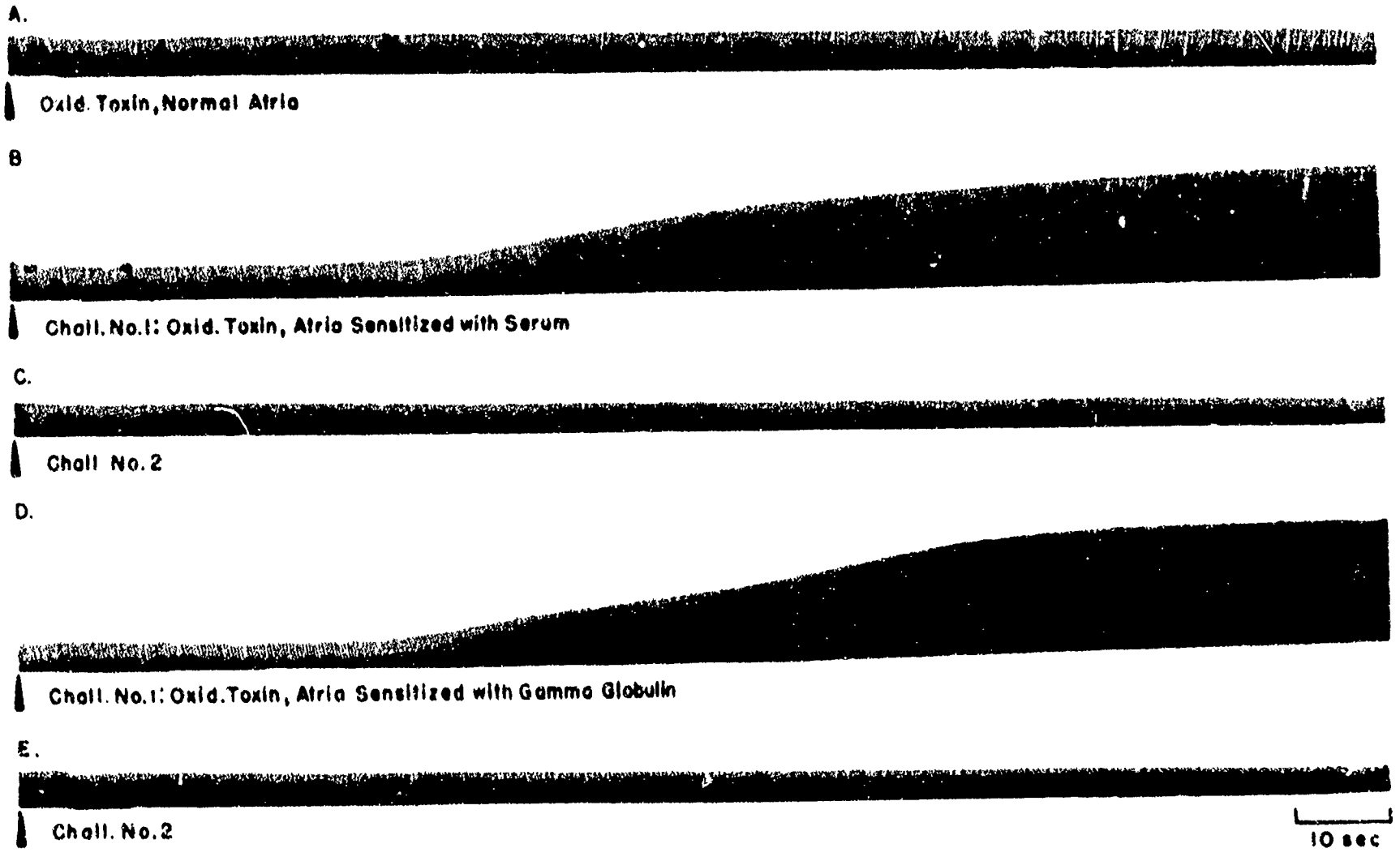
obtained with the oxidized material. The second challenge again demonstrated desensitization of the tissue. Chemical analyses of the bath solutions collected at 3 minutes showed a release of 4.34×10^{-7} moles of histamine/gram dry atria by the first challenge, compared to 0.43×10^{-7} moles by the second challenge; pre-challenge samples as well as the challenge solution from a normal heart challenged with active toxin, were negative for histamine.

Anaphylaxis: Passively Sensitized Tissues.

Experiments were performed to determined if the rabbit anti-streptolysin O antibody was of the type which would sensitize guinea pig tissues. Whole hearts, obtained from normal guinea pigs and prepared and mounted on the Anderson apparatus in the usual manner, were perfused with either 1.0 ml of antiserum (128 ug antibody nitrogen/ml) or 1.0 ml of γ-globulin (37.6 ug antibody nitrogen/ml). In order to avoid the complication of the direct toxic effect of streptolysin O on the whole heart, the tests for anaphylactic sensitization of these hearts were performed on the isolated atria. The atria, dissected from hearts sensitized as described, were mounted in the 3.5 ml muscle baths and challenged with oxidized or reduced toxin.

Figure 32 shows that both the dialyzed serum and the γ-globulin fraction of the serum are capable of sensitizing guinea pig cardiac tissues so that subsequent challenge with the specific antigen results

Figure 39. The anaphylactic response of isolated atria, passively sensitized *in vitro* with either rabbit anti-streptolysin O serum (Traces B and C), or with the γ-globulin fraction (Traces D and E), and challenged with 3.1 mg of oxidized streptolysin O. Bath volume 3.5 ml; 37°C.


A.
Oxid. Toxin, Normal Atria
B
Chall. No.1: Oxid. Toxin, Atria Sensitized with Serum
C.
Chall No. 2
D.
Chall. No.1: Oxid. Toxin, Atria Sensitized with Gamma Globulin
E.
Chall. No.2
10 sec

in an anaphylactic reaction. The data in Table VIII demonstrate that the responses shown in Figure 32 were accompanied by copious histamine release. Both in the atria sensitized with serum, and the atria sensitized with γ-globulin, the first challenge released significant amounts of histamine. No detectable histamine was present in the reaction mixtures from the second challenge, the pre-challenge controls, or the control or challenge solutions from a normal heart exposed to oxidized toxin.

Essentially the same results were obtained when passively sensitized tissues were challenged with reduced streptolysin O. Traces A and B of Figure 33 permit comparison between the response of a normal atrial pair to reduced streptolysin O, and the response of a passively sensitized atrial pair to the reduced toxin. The anaphylactic response is not so severe as expected because of the direct depressant effect of the toxin on the atria. However, the first challenge of the sensitized atria (Trace B) did release histamine, while the normal atria released no detectable histamine (Table IX). The second challenge of the sensitized atria (Trace C) showed no signs of an anaphylactic reaction, and no histamine release was detected by the bioassay.

Because of the unique situation of having two such diverse reactions taking place simultaneously -- the direct action of the reduced toxin on the atria, and the anaphylactic response between the

TABLE VIII

Anaphylaxis in Passively Sensitized Atria Challenged with Oxidized Streptolysin O*

Anaphylactic Reaction					Bioassay**		
Heart No.	Figure No.	*In Vitro* Sensitization	Blocking Agent	Sample	Total Histamine*** (moles/liter)	Dry weight of atria (milligrams)	Histamine Released (M/L/g. dry wt.)
1	32A	none	0	Control	0	238	0
				Chall. 1	0		0
				Chall. 2	0		0
2	32B, C	serum	0	Control	0	209	0
				Chall. 1	6.59×10^{-7}		3.15×10^{-5}
				Chall. 2	0		0
3	32D, E	γ-glob.	0	Control	0	240	0
				Chall. 1	2.36×10^{-6}		9.80×10^{-5}
				Chall. 2	0		0

* 0.1 mg oxidized toxin added to 3.4 ml Chenoweth's solution in bath.

** Bioassay on guinea pig ileum standardized with histamine

*** Total histamine "equivalents" (pyribenzamine-sensitive material) in 1.5 ml bath.

Figure 33. The effect of reduced streptolysin O on normal (Trace A) and passively sensitized (Traces B and C) atria. The effect of 10 mg/ml cysteine on a sensitized atrial preparation (Trace D). 37°C.

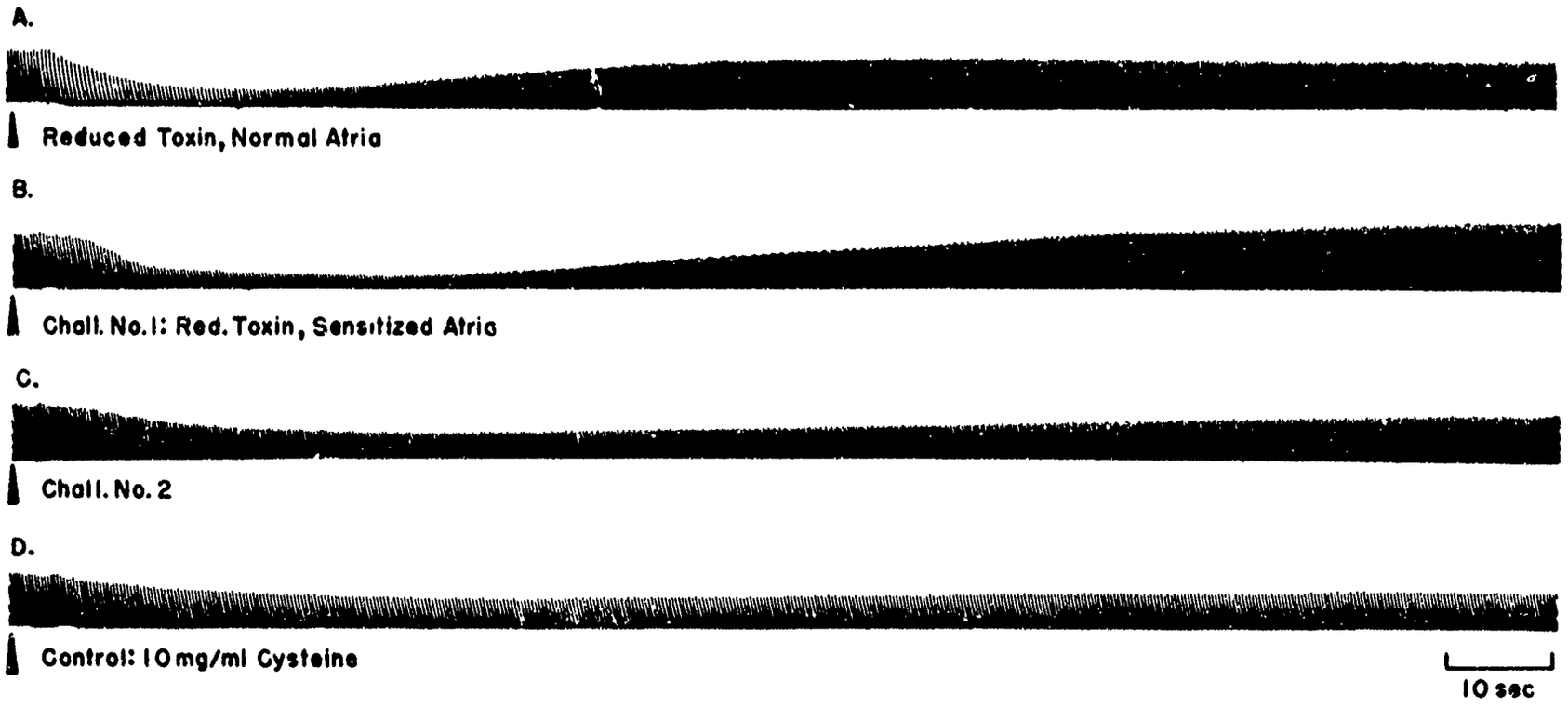

A.
Reduced Toxin, Normal Atria
B.
Chall. No. 1: Red. Toxin, Sensitized Atria
C.
Chall. No. 2
D.
Control: 10 mg/ml Cysteine
10 sec

TABLE IX

Anaphylaxis in Passively Sensitized Atria Challenged with Reduced (Active) Streptolysin O*

Anaphylactic Reaction					Bioassay**		
Heart No.	Figure No.	*In Vitro* Sensitization	Blocking Agent	Sample	Total Histamine*** (moles/liter)	Dry weight of atria (milligrams)	Histamine Released (M/L/g. dry wt.)
4	33A	none	0	Control Chall. 1 Chall. 2	0 0 0	265	0 0 0
5	33B, C 34A	serum	0	Control Chall. 1 Chall. 2	0 9.4 x 10^{-7} 0	205	0 4.9 x 10^{-5} 0
6	34B	serum	Atropine	Control Chall. 1 Chall. 2	0 1.9 x 10^{-6} 0	272	0 7.0 x 10^{-5} 0
7	34C	serum	PBZ	Control Chall. 1 Chall. 2	0 1.2 x 10^{-6} 0	205	0 5.6 x 10^{-5} 0
8	34D	serum	Atropine + PBZ	Control Chall. 1 Chall. 2	0 1.2 x 10^{-6} 0	240	0 4.8 x 10^{-5} 0

* 0.1 mg reduced toxin added to 3.4 ml Chenoweth's solution in bath.

** Bioassay on guinea pig ileum standardized with histamine.

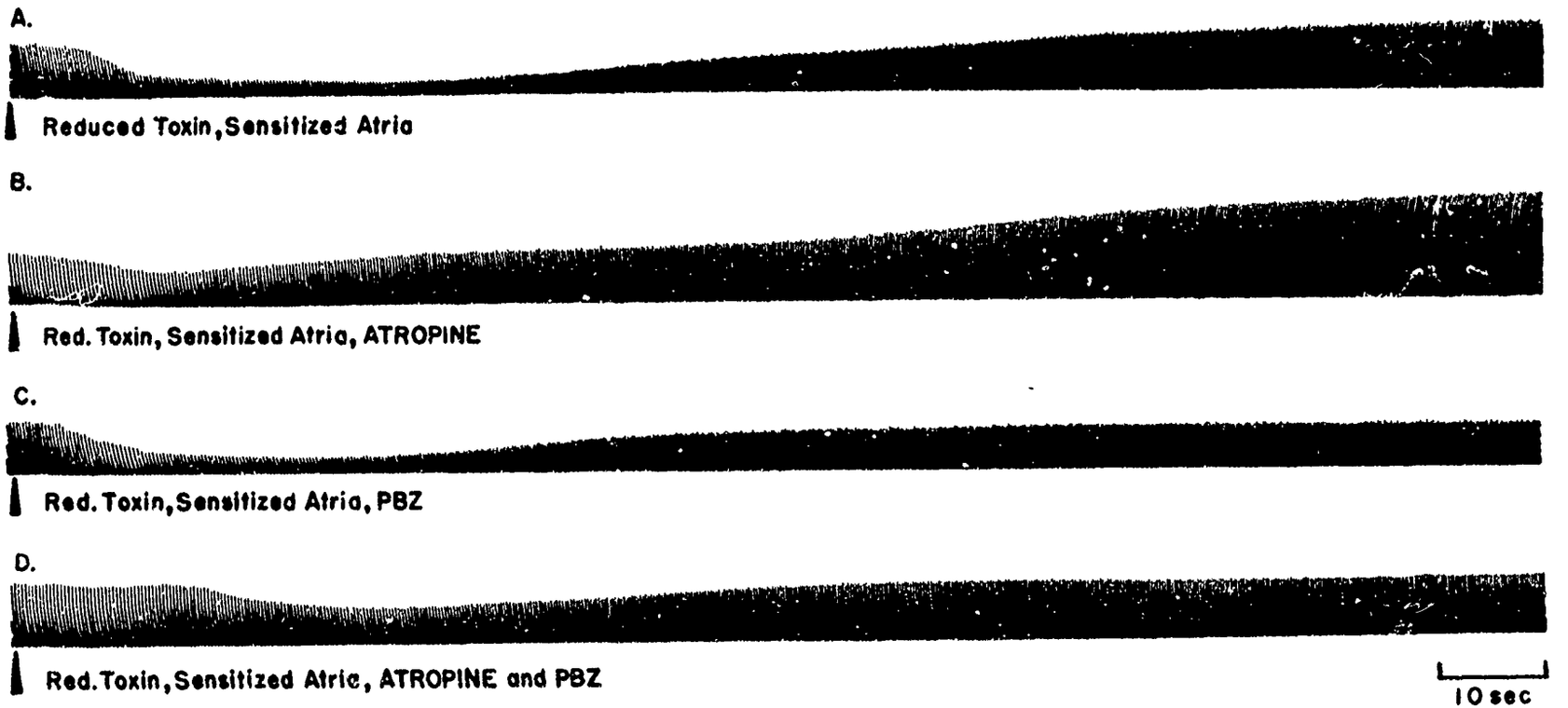
*** total histamine "equivalents" (pyribenzamine-sensitive material) in 3.5 ml bath.

reduced toxin (the antigen) and tissue-bound anti-streptolysin O antibody -- it was necessary to distinguish between the contributions of histamine and those of acetylcholine to the total response, by challenging the atria with reduced toxin in the presence of atropine, pyribenzamine, or both.

The top trace in Figure 34, Trace A, again shows the direct action of the reduced toxin on a normal atrial pair. By blocking the direct depressant effect of the toxin with atropine (see Part I), the anaphylactic response is considerably more pronounced (Trace B). When pyribenzamine is present to block the effect of anaphylactically released histamine on the atria, the anaphylactic response is eliminated, leaving the direct action of the toxin by itself (Trace C). When atropine and pyribenzamine are present together (Trace D), the only effect observed is the slight depressant effect of the cysteine present in the reduced toxin (see cysteine control, Figure 33, Trace D).

The data in Table IX illustrate that, no matter what blocking agents were present to modify the observed mechanical changes, histamine was released by the first challenge with the reduced toxin; the pre-challenge and second challenge samples released no detectable histamine.

Figure 34. The effects of pyribenzamine (0.2 µg/ml) and atropine (8.22×10^{-7} *M*) on the response of passively sensitized atria to reduced streptolysin O. 37°C.


A.
Reduced Toxin, Sensitized Atria
B.
Red. Toxin, Sensitized Atria, ATROPINE
C.
Red. Toxin, Sensitized Atria, PBZ
D.
Red. Toxin, Sensitized Atria, ATROPINE and PBZ
10 sec

DISCUSSION

The experiments comprising Part III confirm the fact that streptolysin O is antigenic and demonstrate conclusively that the oxidized form of the toxin is capable of eliciting the production of homocytotropic anaphylactic antibodies in the guinea pig, as well as heterocytotropic antibodies in the rabbit which can passively sensitize guinea pig tissues.

The classical Schultz-Dale reaction was used to show that ileal tissue from a guinea pig immunized with oxidized streptolysin O was indeed sensitized and would contract when challenged with the sensitizing antigen (Figure 29A). That this response was due to a specific anaphylactic reaction was confirmed by the failure of the desensitized ileum to respond to a second challenge and by the lack of a response when the oxidized toxin was tested on a control, unsensitized, ileum (Figure 29B).

Whole hearts and isolated atrial pairs from actively sensitized guinea pigs were tested for their response to both the oxidized and reduced toxins. The whole heart reacted characteristically when challenged with the oxidized material, and histamine was quantitatively recovered from the perfusate, during the response. The usual manifestations of the anaphylactic

response in the guinea pig heart -- increased rate and amplitude of contraction followed by partial atrio-ventricular block -- were reproduced by a suitable dose of histamine (Figure 30), (70). The desensitized heart failed to respond to a second challenge.

To eliminate the complicated, direct, effects of streptolysin O on the whole heart, the remaining experiments were performed on isolated atria obtained from sensitized whole hearts. Using this technique, a strong response was seen when the sensitized atria were challenged with oxidized toxin. Concomitantly, there appeared large amounts of histamine in the reaction fluid. It was shown additionally that the sensitized tissue also reacted anaphylactically when challenged with the active (reduced) toxin (Figure 31). This demonstration, then, confirmed the results of Neill and Mallory (19), who found that the two forms of the toxin were antigenically indistinguishable.

We have demonstrated that rabbits, appropriately injected with oxidized streptolysin O, produce an anti-streptolysin O antibody which is capable of passively sensitizing guinea pig tissues, *in vitro*. The serum obtained from such rabbits, as well as the γ-globulin fraction of this serum, passively sensitized guinea pig hearts. The atria from these hearts were shown to react vigorously, and to release histamine when tested with either

the oxidized or reduced toxin preparations (Figure 32, 33, 34, Tables VIII, IX).

The reaction between sensitized atria and the active streptolysin O showed a pronounced bi-phasic response. There was an initial depression of atrial contraction due to the direct action of the toxin on the tissue. This depression was followed by a gradual increase in the rate and amplitude of contraction which exceeded the usual post-response potentiation.

The components of this biphasic response were resolved by the use of the specific blocking agents, atropine and pyribenzamine. When sensitized atria were challenged with reduced toxin in the presence of atropine, the depressant effects of the active toxin were blocked, and the potentiating effects of the anaphylactically released histamine (Table IX) were much more pronounced (Figure 34, Trace B). Just the opposite occurred when sensitized atria were challenged in the presence of pyribenzamine. The anaphylactic phase of the reaction was blocked, so that only the direct, depressant effect of the active toxin on the tissue was observed (Figure 34C). When sensitized atria were challenged in the presence of both agents, both phases of the reaction were blocked (Figure 34D).

It can be concluded from these results that a toxic protein, such as streptolysin O, can exhibit multiple reactions with cardiac

tissues. The direct action of the toxin is, in itself, a dual one: it is made up of a reversible depressant effect on atrial contraction due to the release of acetylcholine, and of an irreversible effect owing to an unspecified conduction defect resulting in ventricular standstill. The "passive" action of the toxin results from its immunogenic properties as a protein. The reaction between the toxin (in either the active or inactive form) and the tissue-fixed, anti-streptolysin O antibodies may lead to focal lesions, due to the presence of the antigen-antibody complex; or to tissue damage resulting from the direct action of vasoactive materials, such as histamine, released as a consequence of the antigen-antibody reaction.

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13. ABSTRACT

The direct cardiotoxicity of streptolysin O (SLO) may be aggravated by an additional hypersensitivity reaction between SLO and specific, tissue-bound antibody.

The isolated guinea pig heart responds to purified SLO with a rapid, transient decrease in rate and amplitude of contraction, superimposed on a gradual, irreversible loss of ventricular contractility. At ventricular standstill, the atria beat normally, as do electrically driven strips of the arrested ventricles. Ventricle strips from normal hearts are completely insensitive to the toxin. However, isolated atria respond to SLO with a reversible decrease in rate and amplitude of contraction accompanied by a marked, transient increase in the rate of repolarization of the intracellular potential. All of the transient changes are shown to be due to acetylcholine release by the atria; the permanent ventricular decline results from a toxin-induced defect in atrio-ventricular conduction.

Oxidized (inactive) SLO elicits the production of anaphylactic antibodies in the guinea pig, and when challenged with the oxidized or reduced (active) toxin, whole hearts and isolated atria from these animals respond anaphylactically and release histamine. Serum from rabbits immunized with the oxidized toxin passively sensitizes guinea pig cardiac tissues *in vitro*. These tissues, when challenged with either form of SLO, react anaphylactically and release histamine. The response of sensitized atria to reduced toxin consists of the depression of the direct toxic effect (acetylcholine), followed by the potentiation of the anaphylactic reaction (histamine).

Thus, SLO can participate in multiple reactions with cardiac tissues. The pathologic significance of these findings with respect to human disease is discussed.

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5. RHEUMATIC FEVER						
6. RHEUMATIC CARDITIS						
7. CARDIAC LESIONS OF RHEUMATIC CARDITIS						
8. ANAPHYLAXIS						
9. CARDIAC ANAPHYLAXIS						
10. ANAPHYLACTIC HISTAMINE RELEASE						
11. HISTAMINE						